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Immune escape is a crucial feature of cancer progression about which little is known. Elevation of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) in tumor cells can facilitate immune escape. Not known is how IDO becomes elevated or whether IDO inhibitors will be useful for cancer treatment. Based on evidence that Bin1 loss elevates IDO and enhances immune evasion by tumor cells, we hypothesized that reversing IDO activity with chemical inhibitors would enhance immune recognition and rejection of tumor cells. We aimed to (1) generate an antibody to IDO to assess its expression in normal and malignant breast tissue, (2) identify pharmacologically attractive 'lead' inhibitors of IDO, and (3) investigate the ability of new compounds with IDO inhibitory activity to block tumor growth in the MMTV-neu transgenic mouse model of breast cancer. In Year 2 of this project all Aims as proposed originally are essentially complete. This year we report new bioactive inhibitors, development of a second model for efficacy testing, and compound formulation studies. Two important outcomes this year were publication of the core findings of our study in *Nature Medicine* and funding of an NIH grant to drive medicinal chemistry and drug development of new lead inhibitors.

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2. Muller, A.J., Malachowski, W.B., and Prendergast, G.C. (2005). IDO in cancer: targeting pathological immune tolerance with small molecule inhibitors. Exp. Opin. Ther. Targets, in press.

Introduction

Immune escape is a fundamental trait of cancers including breast cancer. While there is great interest in ways to stimulate immune rejection of breast cancers, no effective strategies to achieve this end have been identified. Such strategies are particularly appealing for the treatment of systemic disease, which constitutes the major clinical challenge. We have identified in the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) an attractive and tractable target for development of drugs to treat breast cancer. IDO has been implicated in immunosuppression but its possible role in cancer has received little attention to date. Recent work has revealed that IDO is overexpressed commonly in human cancers. Moreover, it has been shown that IDO overexpression can contribute significantly to immune escape by tumor cells. We identified IDO through its genetic interaction with Bin1, a cancer suppression gene discovered in our laboratory. A large body of evidence argues that Bin1 acts to facilitate stress signaling and to restrain malignant development[1-9]. Loss of Bin1 expression occurs frequently in breast tumors[7,9]. Our studies in a knockout mouse model have revealed that Bin1 loss leads IDO-mediated immune escape of neoplastically transformed cells. Since Bin1 loss elevates IDO, which suppresses immunity, we hypothesized that chemical inhibitors of the IDO would relieve breast tumor immunosuppression and elicit breast tumor cell death.

Body

Our proposal to the DoD Breast Cancer Program included three aims, as follows, all of which are now complete within the scope of the original application. This year we published at *Nature Medicine* a summary of the work completed on the DoD grant to date[10]. A reprint of this report is included in the Appendix, along with a recent review now in press from the group in the Appendix (please refer to reprint for experimental results).

Aim 1. Examine IDO expression in normal and malignant breast tissues. Monoclonal antibodies to human or mouse IDO were to be raised and used for analysis of breast tissues derived from human patients or MMTV-neu mice.

Progress. This aim was completed last year. The IDO monoclonal antibody we created was licensed to Chemicon and UBI to make it available to the general research community. Recent information from Chemicon has indicated that in the last six-month period the antibody has generated $20K in sales, indicating that the antibody is addressing an important gap in the field. We are pleased with the properties of this antibody but are considering generating a second to improve sensitivity for tissue staining applications, which we think will be most important to researchers. The present antibody that was commercialized is described at http://www.chemicon.com/Product/ProductDataSheet.asp?TXTSEARCH=IDO&ProductItem=MAB5412.

Aim 2. Identify lead inhibitors of IDO that have better potency, pharmacodynamics, and pharmacokinetic properties than 1-methyl-tryptophan (1MT), an existing inhibitor of IDO. Biochemical and cell-based assays were to be used to identify novel IDO inhibitors among commercially available compounds.

Progress. This aim is complete within the scope of the original proposal. This year we continued to drive development of drug-like inhibitors in several ways, including:

- Continued work through our RAND award to screen the NCI compound collection (described at http://dtp.nci.nih.gov/docs/rand/randwin6.html). Early this year the NCI team indicated that it had overcome technical difficulties met during the year in micronizing the IDO inhibitor screen to a 384-well assay (needed because the NCI compound collection is plated in this format). A training set is currently being run. In the coming year, we hope to receive the first 'hits' that are identified by this team.

• A new NIH R01 grant was awarded to our consortium group to develop IDO inhibitors for combinatorial cancer treatment (CA 109542). This grant aims to perform medicinal chemistry on 'lead' inhibitors that had been identified by screening commercially available compounds (Sigma Aldrich), under the auspices of the DoD grant. The medicinal chemistry is performed in collaboration with Dr. William Malachowski's group in the Department of Chemistry at Bryn Mawr College. PK/PD and efficacy assays that continue to be refined and developed under the auspices of the DoD award will be used for screening compounds that have potent IDO inhibitory activity in vitro and in cells.

• A pharmaceutical partnership with the start-up company (OncoRx, Inc.) that had out-licensed the original composition of matter and methods patents resulting from our work at LIMR. The goal of this partnership is to perform required preclinical toxicology and other development of lead compounds to allow them to move them appropriately to Phase I/IIa clinical testing. The pharmaceutical company has agreed to dedicate $2M to development of a lead candidate for clinical testing.

The goal of Aim 2 was to identify 'lead' inhibitors of IDO that have better potency, pharmacodynamics, and pharmacokinetic properties than 1-methyl-tryptophan (1MT), an existing inhibitor. As described last year, this effort had led to the identification of 'lead' structural series including the thiohydantoin derivatives of tryptophan (see reprint). Three natural products known to have anticancer properties were also identified in namely the cruciferous phytochemicals indoleamine-3-carbinol (I3C) and 3,3-diindolylmethane (DIM) and the plant phytoalexin 3-(S-methyldithiocarbamoyl)-aminomethyl indole, known as brassinin[11-14]. This year, by analysis in the in-cell IDO inhibitor assay, the PK assay, and the MMTV-neu efficacy assay (see reprint), we found that:

• I3C did not inhibit IDO activity in cells.

- DIM inhibited IDO in cells but it was inactive in animals at the highest concentration tested (1000 mg/kg/day).
- Brassinin was active in both the in-cell and efficacy assay.

Further screening identified ß-carbinol and 3-amino-2-naphthoic derivatives of tryptophan as IDO inhibitors. Based on the results obtained, we proposed in the NIH grant application to perform medicinal chemistry to derivatize and test a total of four series of IDO inhibitors, including the thiohydantoin, ß-carbinol, and 3-amino-2-naphthoic derivatives of tryptophan and the brassinin derivatives. Ongoing work with Dr. Malachowski's group has revealed significant in vivo potency of 5-bromo-brassinin and several derivatives of this compound, with potencies in the low micromolar range. We are currently focusing on these comounds. In the coming year, under the auspices of the DoD grant we will try to crystallize IDO with one or more of the new inhibitors, for structural studies to aid drug design. We also aim to further refine the pharmacodynamic assay for assessing in vivo potency, and to explore formulation of IDO inhibitors in ß-cyclodextrin, used commonly to increase drug exposure in animals. Preliminary experiments suggest that ß-cyclodextrin can greatly enhance exposure of brassinin derivatives.

Aim 3. Determine whether systemic inhibition of IDO in MMTV-neu mice could promote immune infiltration and rejection in MMTV-neu mice, a well-established transgenic mouse model of breast cancer.

Progress. The aim is complete within the scope of the original proposal. During the remainder of the award period, we have expanded the scope of the aim to further characterize and examine the mechanism of our discovery that systemic inhibition of IDO enhances the efficacy of a variety of classical chemotherapeutic agents (see reprint). In current work, we are developing 'classical' models beyond the MMTV-neu model for the purpose of efficacy testing and mechanistic analysis. In one arm of the work, we are working to adapt the 4T1 breast cancer model performed in BALB/c mice, using luciferase-tagged cells that can allow

quantitative tumor imaging *in vivo* using bioluminescence (our Institute has an appropriate bioluminscence imaging device which we have been trained on by an expert LIMR faculty collaborator [J. Sawicki]). One goal is to compare efficacy in growth inhibition versus regression assays, where therapy is given at the time of tumor cell introduction versus after formation of a localized tumor. 4T1 is particularly aggressive and very metastatic, more so than the MMTV-neu model, so we believe that the 4T1 model will give additional insight into the effectiveness of the IDO inhibitors at limiting disseminated cancers (which are typically the cause of demise in the 4T1 model). In preliminary work we are encouraged by responses to IDO combination therapy with cyclophosphamide, a standard robust therapy in 4T1 model, which include an apparent cure that is not seen in this model with cytotoxic therapy alone. In a second arm of the work, we are expanding immune cell depletion experiments in an effort to define the mechanism(s) by which IDO inhibition leverages chemotherapeutic efficacy. Currently, we have obtained evidence of a role for CD4+ and CD8+ T cells in the mechanism[10], as expected, but we are now aiming to explore possible roles for CD25+ T regulatory cells and various classes of antigen-presenting dendritic cells and myeloid suppressor cells.

Key Research Accomplishments

1. Our study of the discovery of IDO regulation by the cancer suppression gene Bin1 and the antitumor efficacy of IDO inhibitors, alone and in combination with classical chemotherapeutics, was published in the March 2005 issue of *Nature Medicine* (Muller, A.J., DuHadaway, J.B., Donover, P.S., Sutanto-Ward, E., and Prendergast, G.C. [2005]. Inhibition of indoleamine 2,3-dioxygenase, a target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat. Med. 11: 312-319) (see reprint in Appendix). Our group currently has two reviews of this work and its relationship to the IDO literature that are accepted or in press at *Cancer Research* and *Expert Opinion in Therapeutic Targets.*

2. The natural product brassinin was found to be a potent in vivo inhibitor of IDO. This finding may offer an explanation for its anticancer mechanism[14]. From this lead, 5-bromo-brassinin was found to be a more active inhibitor of IDO, with an IC50 of ~20 μM. Preliminary sutides suggest this compound is active in vivo and that it has translational potentical. Derivatives of this compound are currently being explored.
3. Novel inhibitors of IDO were identified in the thiohydantoin and brassinin structural series.
4. The 4T1 breast cancer model was introduced for tumor biology studies.

Reportable Outcomes

1. Publication of the discovery that the Bin1-IDO pathway defines a mechanism for immune escape in cancer, and that IDO inhibitors can be used in combination with chemotherapy to improve antitumor efficacy.
2. Four distinct structural series of novel bioactive inhibitors of IDO have been identifed, including the natural product brassinin and the thiohydantoin, ß-carbinol, and 3-amino-2-naphthoic derivatives of tryptophan.
3. NIH funding has been obtained to drive medicinal chemistry and preclinical drug development in these 'lead' series discovered under the auspices of the DoD award.

Conclusions

The original aims of the the proposal – to gain support for the general hypothesis that IDO inhibitors offer a novel modality to enhance breast cancer therapy – are complete. A major publication on the work was communicated this year in *Nature Medicine* and NIH funding has been received to drive the work, leveraging the value of the DoD funds that have been obtained. Aim 1 may be extended to generate a second IDO monoclonal antibody that is selected specifically for tissue staining applications which we believe will be tantamount to the field. Aim 2 is being extended to explore crystallization and formulation of bioactive compounds. We will also continue to work this year with the NCI

group as it identifies 'hits' from their compound collection, to assess bioactivity and pharmacology and possibly to explore derivitization of any 'lead' compounds identified (with the goal of enhancing potency and PK/PD characteristics). Aim 3 is being extended to explore the 4T1 mouse model of breast cancer, which is particularly metastatic, as a means to further characterize the efficacy of IDO inhibitors. Additionally, we are performing additional immune cell depletion experiments in the MMTV-neu model to focus on the precise mechanism by which IDO inhibition enhances cancer chemotherapy.

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Appendix

Muller, A.J., DuHadaway, J.B., Donover, P.S., Sutanto-Ward, E., and Prendergast, G.C. (2005). Inhibition of indoleamine 2,3-dioxygenase, a target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nature Med. **11,** 312-319.

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nature medicine

Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene *Bin1*, potentiates cancer chemotherapy

Alexander J Muller[1], James B DuHadaway[1], P Scott Donover[1], Erika Sutanto-Ward[1] & George C Prendergast[1,2]

Immune escape is a crucial feature of cancer progression about which little is known. Elevation of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) in tumor cells can facilitate immune escape. Not known is how IDO becomes elevated or whether IDO inhibitors will be useful for cancer treatment. Here we show that IDO is under genetic control of *Bin1*, which is attenuated in many human malignancies. Mouse knockout studies indicate that *Bin1* loss elevates the STAT1- and NF-κB-dependent expression of IDO, driving escape of oncogenically transformed cells from T cell–dependent antitumor immunity. In MMTV-*Neu* mice, an established breast cancer model, we show that small-molecule inhibitors of IDO cooperate with cytotoxic agents to elicit regression of established tumors refractory to single-agent therapy. Our findings suggest that *Bin1* loss promotes immune escape in cancer by deregulating IDO and that IDO inhibitors may improve responses to cancer chemotherapy.

Immune cells create a complex cytokine environment that promotes cancer cell survival, angiogenesis, invasion and metastasis[1]. To survive in this environment, however, cancer cells expressing recognizable tumor antigens must evolve strategies to thwart immune detection and destruction[2]. Immune escape is thus a hallmark of cancer progression, but its underlying molecular genetic basis remains poorly understood. The interplay between immune escape and other hallmarks of malignant conversion, such as invasion and metastasis, is similarly obscure. Aggressive and disseminated cancers can be eradicated by an appropriately activated immune system, arguing that overcoming immune escape might have broad therapeutic impact, but this expectation has yet to be realized. Small-molecule drugs are of particular interest because of their relative advantages compared to biological agents in terms of production, delivery and cost. Yet few small molecules for stimulating antitumor immunity have been described.

Studies of the BAR adapter–encoding gene *Bin1* (also known as *Amphiphysin2*) indicate that it functions in cancer suppression[3–9]. Certain Bin1 adapter isoforms associate with endocytotic complexes[10], but evidence from gene knockouts in several species suggest that *Bin1* is not essential for endocytosis[11–13]. Instead, Bin1 adapters may be important for vesicle trafficking[14], consistent with evidence that BAR domains can act as sensors of membrane curvature[15]. BAR adapter proteins may integrate signaling and trafficking processes, in some cases perhaps involving sites of action in the nucleus[4,16]. Nuclear localization of some Bin1 isoforms is important for cancer suppression; however, there is little information about the relevant effector pathways or about the precise pathophysiological consequences of attenuating the expression of nuclear isoforms, as occurs often in human malignancies[3,5–8].

We report that Bin1 is involved in controlling expression of the *Indo* gene, which encodes the IDO enzyme. IDO is emerging as an important immunoregulatory enzyme. It catalyzes the initial rate-limiting step in tryptophan catabolism, which leads to the biosynthesis of nicotinamide adenine dinucleotide. By depleting tryptophan from local microenvironments, IDO can block activation of T lymphocytes, which are particularly sensitive to loss of this essential amino acid[17,18]. Notably, IDO is needed to prevent T cell–mediated rejection of allogenic concepti[19]. IDO is overexpressed in many cancers, where it has been implicated in immune escape[20,21]. But its importance to cancer progression and therapy has yet to be gauged fully. Here we identify a mechanism for IDO elevation in cancer, and we show how pharmacological inhibitors of IDO can be used in combination with cytotoxic chemotherapeutic agents to elicit regression of established tumors.

RESULTS

Bin1 loss promotes immune escape by tumor cells

Based on evidence of interaction between Bin1 and c-Myc (encoded by *Myc*)[3,4,9,22], we investigated the effects of targeted deletion of the *Bin1* gene[12] on the malignant phenotype of primary mouse skin epithelial cells (keratinocytes) cotransformed by *Myc* plus an activated allele of *Hras1* (these cells are referred to below as MRKECs). We confirmed *Bin1* genotype and transgene expression by PCR analysis and western blot analysis respectively (**Fig. 1a**). *Bin1* deletion did not alter the phenotype

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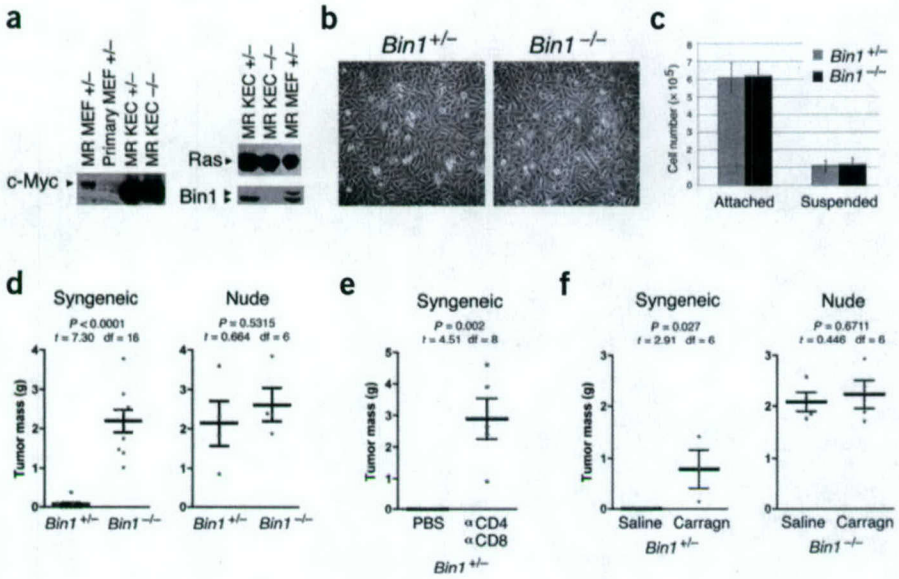


Figure 1 *Bin1* loss promotes tumor formation by facilitating immune escape. (**a**) Western blot analysis of MRKECs. (**b**) Cell morphology. (**c**) Cell proliferation. The experiment was performed twice. (**d**) Tumor formation in syngeneic versus nude mice. Tumor weight was determined 4 weeks after subcutaneous injection of MRKECs into syngeneic or nude mice. Each point on the graph represents a single tumor measurement with mean and standard error shown for each group ($t = t$ ratio (the difference between sample means divided by the standard error of the difference between the means); df = degrees of freedom ($n - 2$)). (**e**) Immune cell depletion phenocopies *Bin1* loss. $Bin1^{+/-}$ MRKECs were injected subcutaneously into mice depleted of both $CD4^+$ and $CD8^+$ cells and tumor formation was scored 3 weeks later. PBS, phosphate-buffered saline. (**f**) Carrageenan (Carragn) treatment partly phenocopies *Bin1* loss. $Bin1^{+/-}$ MRKECs were injected subcutaneously into control (saline-treated) and carrageenan-treated mice and tumor formation was scored 4 weeks later. *n* for all animal experiments can be calculated by using the formula $n = \text{df} + 2$.

or *in vitro* proliferation of MRKECs under either anchorage-dependent or anchorage-independent conditions (**Fig. 1b,c**). Nevertheless, *Bin1* loss substantially enhanced the outgrowth of tumors formed by MRKECs in syngeneic animals (**Fig. 1c**). The significant difference in tumorigenicity between $Bin1^{-/-}$ and $Bin1^{+/-}$ cells ($P < 0.0001$) could not be explained by increased intrinsic cell proliferation, because $Bin1^{+/-}$ cells were no less aggressive at forming tumors than $Bin1^{-/-}$ cells in T cell–deficient, athymic nude mice (**Fig. 1d**) or in syngeneic mice depleted of both $CD4^+$ and $CD8^+$ T cells (**Fig. 1e**). Because tumor cells present antigens in part by cross-priming of antigen-presenting cells (APCs), we investigated the effect of *Bin1* loss on tumors formed in syngeneic mice treated with carrageenan, a substance that depletes or inactivates phagocytic cells including macrophages, dendritic cells and neutrophils[23]. Carrageenan treatment enhanced tumor formation by $Bin1^{+/-}$ cells but not by $Bin1^{-/-}$ cells (**Fig. 1f**), consistent with the idea that cross-priming is involved in the antitumor immune response. In summary, we conclude that *Bin1* suppresses tumor formation through a cell-extrinsic, immune-based mechanism that appears to be dependent on both T cells and APCs.

IDO is under genetic control of *Bin1*

The expression of tumor antigens by most cancers means that they must evolve mechanisms to escape or subvert antitumor immunity in order to progress successfully. Two recent studies have suggested that *Bin1* functions may modulate subcellular trafficking of the STAT and NF-

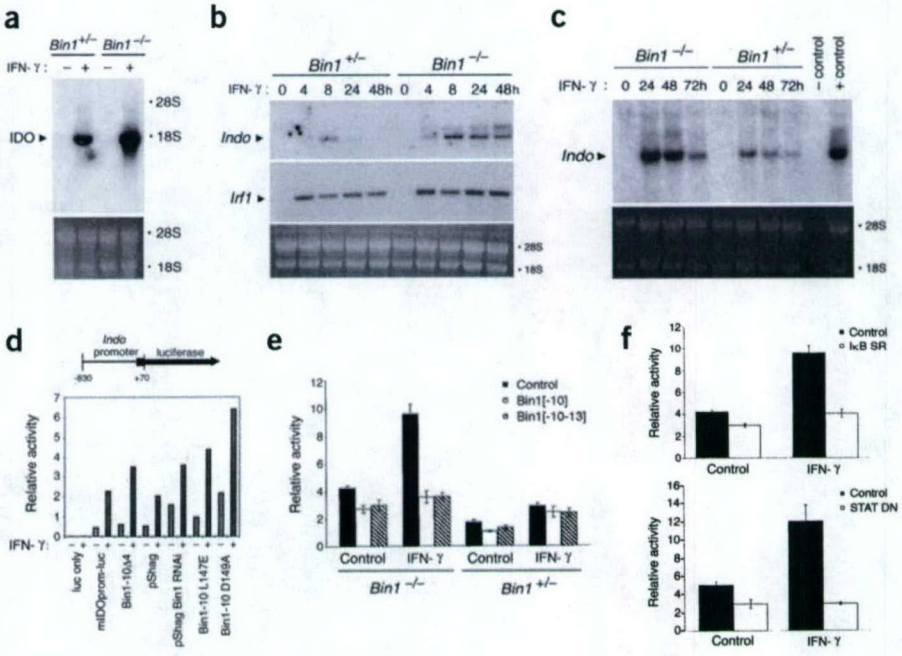


Figure 2 *Bin1* loss potentiates the NF-κB- and STAT-dependent expression of *Indo*. (**a**) *Bin1* loss elevates *Indo* expression in MRKECs treated with IFN-γ for 24 h. Steady-state RNA was analyzed by northern blotting with a mouse *Indo* cDNA probe. Ethidium bromide–stained gel is shown below as an RNA loading control. (**b**) *Bin1* loss leads to persistent induction of *Indo*. MRKECs were treated with IFN-γ for times indicated and processed for northern blot analysis as above. (**c**) *Bin1* loss accentuates induction of *Indo* in *Myc*-immortalized macrophages. Cells were treated with IFN-γ for times indicated and processed for northern blot analysis as before. (**d**) *Bin1* attenuation potentiates *Indo* transcription in human cells. HeLa cells were transfected with a mouse *IDO* promoter-luciferase reporter plus the indicated expression vectors. The day after transfection, cells were either left untreated or treated 16 h with IFN-γ and cell extracts were processed for normalized luciferase activity. (**e**) *Bin1* deletion potentiates *Indo* transcription in MRKECs. Cells were transfected with the *Indo* promoter reporter used above plus the indicated expression vectors, then treated and processed for normalized luciferase activity as before. (**f**) NF-κB and STAT1 are required for superinduction of *Indo* in *Bin1*-null cells. $Bin1^{-/-}$ MRKECs were cotransfected with *Indo* promoter-reporter used above plus the indicated expression vectors, then treated and processed for luciferase activity as before.

κB transcription factors, which have important roles in modulating immunity[9,24]. In considering common genetic targets of STAT and NF-κB, we identified IDO (encoded by *Indo*) as a candidate that might explain the effects of *Bin1* loss on immune escape. IDO is an extrahepatic oxidoreductase. In APCs, the expression of IDO is strongly elevated by interferon-γ (IFN-γ)[17,25]. Deletion of *Bin1* in MRKECs markedly increased IFN-γ-induced expression of IDO both quantitatively and temporally, such that the *Indo* message level was both higher and persisted longer than in *Bin1*-expressing cells (**Fig. 2a,b**). IDO was also superinduced by IFN-γ in *Bin1*$^{-/-}$ macrophages[12], showing that the *Bin1* deletion has a similar effect on IDO expression in a cell lineage in which IDO activity is known to be physiologically relevant (**Fig. 2c**). Transcription assays in human HeLa cells showed that attenuation of *Bin1* by either siRNA or dominant inhibitory strategies increased basal and IFN-γ-induced activity of the *Indo* promoter (**Fig. 2d**). Similarly, *Bin1* deletion elevated basal and IFN-γ-induced activity of the *Indo* promoter in MRKECs, and these effects were reversed by ectopic expression of cDNAs encoding the two ubiquitously expressed Bin1 splice isoforms *Bin1[-10]* or *Bin1[-10-13]* cDNAs encoding the two Bin1 proteins that are ubiquitously expressed (**Fig. 2e**). Activation of the *Indo* promoter requires the activity of STAT1 and NF-κB transcription factors[26], the regulation of which may be influenced in part by Bin1 functions[9,24]. We observed that the benefits of *Bin1* loss to IDO transcription were abolished by introduction of a 'super-repressor' mutant of IκB, which prevents NF-κB activation, as well as by a dominant negative STAT1 mutant (STAT1 Y701F), which prevents STAT1 activation (**Fig. 2f**). Taken together, these results indicate that *Indo* expression is under the genetic control of *Bin1* at the level of NF-κB- and STAT1-dependent transcription.

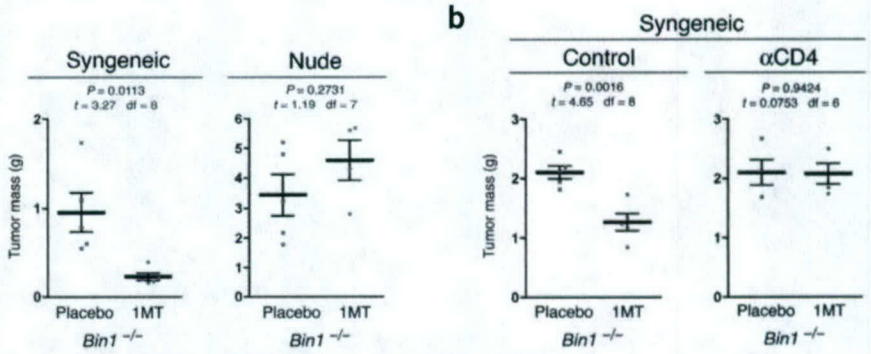


Figure 3 IDO inhibition counteracts the benefit of *Bin1* loss to tumor formation. (**a**) 1MT inhibits tumor formation by *Bin1*$^{-/-}$ cells. Mice implanted with either 1MT or placebo time-release pellets were injected subcutaneously with *Bin1*$^{-/-}$ MRKECs and tumor formation was scored 2 weeks later. (**b**) 1MT activity is abolished by T-cell depletion. Tumor formation was initiated and scored as before after subcutaneous injection of *Bin1*$^{-/-}$ MRKECs into mice treated with phosphate-buffered saline or CD4-specific monoclonal antibodies. n = df + 2.

IDO mediates immune escape by tumor cells that lack *Bin1*

To determine the importance of IDO activity to immune escape caused by *Bin1* loss, we asked whether the IDO inhibitor 1-methyl-DL-tryptophan (1MT) could specifically counteract the benefit of *Bin1* loss to MRKEC tumor growth in syngeneic mice. We confirmed that the delivery method used (subcutaneous time-release pellets) could elicit maternal immune rejection of allogeneic but not syngeneic concepti (**Supplementary Fig. 1** online), indicating sufficient systemic exposure of 1MT to achieve biological activity. In tumor-bearing animals, 1MT inhibited the growth of *Bin1*$^{-/-}$ tumors in syngeneic hosts but not in athymic nude mice

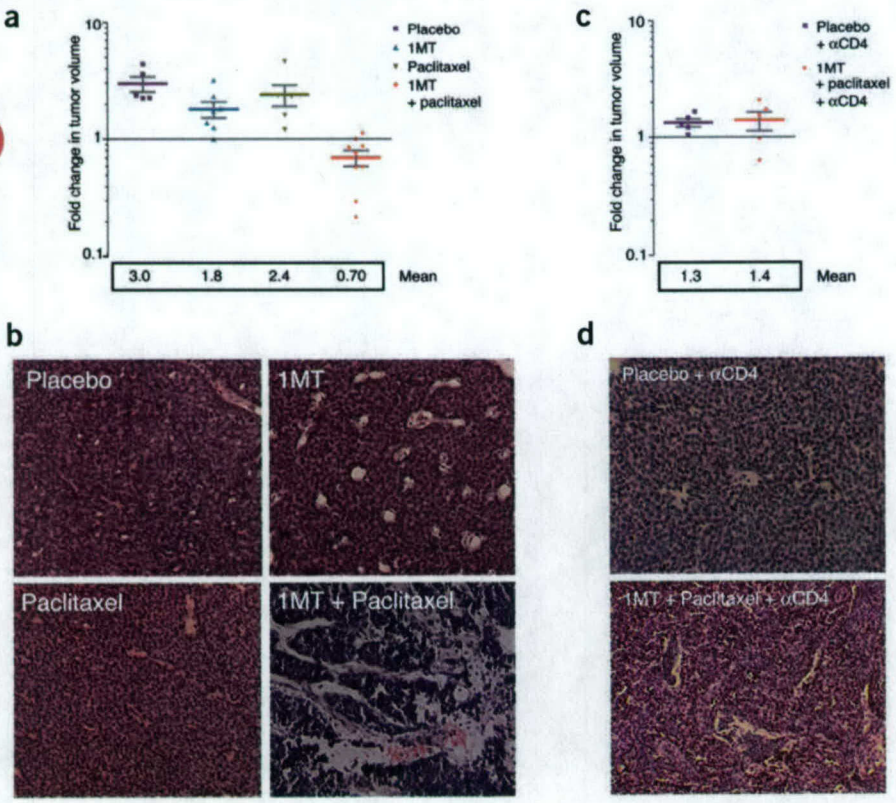


Figure 4 IDO inhibition cooperates with paclitaxel to cause regression of autochthonous MMTV-*Neu* breast tumors. (**a**) Therapeutic response. Tumor-bearing MMTV-*Neu* mice were implanted with time-release pellets containing 1MT (20 mg/d) or placebo pellets. The next day, either paclitaxel (13.3 mg/kg [MTD]) or vehicle was delivered three times per week as an intravenous bolus dose. Tumor volumes were calculated 2 weeks after therapy was initiated. Each point represents the fold change in volume for an individual tumor with the mean ± s.e. indicated for each group (n = 5,7,6,9 in the order graphed). (**b**) Tumor histology at endpoint. Representative hematoxylin and eosin–stained sections were photographed using a 10x objective on an Olympus BH2 microscope. (**c**) Immune depletion abolishes the efficacy of the combination therapy. Tumor-bearing mice depleted of CD4$^+$ cells were treated with either combination therapy or vehicles and tumor volumes were calculated 2 weeks later (n = 5,5 in the order graphed). (**d**) Tumor histology at endpoint in immune-depleted mice. Photographs were taken as in **b**.

Table 1 IDO inhibition enhances the efficacy of certain commonly used cancer chemotherapeutic agents

Compound	Class	Mean ± s.e. (+1MT)	Mean ± s.e. (–1MT)	*P*	*n*	Dose (mg/kg)	Route	Schedule
Cisplatin	Alkylating agent	0.77 ± 0.18	1.7 ± 0.33	0.0419	7,8	1.0	i.v.	3×/week
Cyclophosphamide	Alkylating agent	0.81 ± 0.12	1.4 ± 0.18	0.0269	5,5	100	i.v.	3×/week
Doxorubicin	Antineoplastic antibiotic	0.79 ± 0.07	1.5 ± 0.25	0.0150	6,4	0.66	i.v.	3×/week
5-Fluorouracil	Antimetabolite	1.2 ± 0.20	1.1 ± 0.25	0.8926	8,7	50	i.v.	3×/week
Methotrexate	Antimetabolite	1.7 ± 0.28	1.7 ± 0.38	0.9047	3,3	1.0	i.v.	3×/week
Paclitaxel	Mitotic inhibitor (taxane)	0.68 ± 0.11	2.4 ± 0.43	0.0010	8,7	13.3	i.v.	3×/week
Vinblastine	Mitotic inhibitor (vinca alkaloid)	1.3 ± 0.19	1.2 ± 0.18	0.7368	10,8	1.0	i.v.	3×/week
FTI	Signal transduction inhibitor	0.67 ± 0.11	1.0 ± 0.16	0.0979	8,8	40	i.p.	qdx11
Rapamycin	Signal transduction inhibitor	0.97 ± 0.07	0.99 ± 0.25	0.9417	4,4	1.5	i.v.	qdx11
Tetrathiomolybdate	Antiangiogenic (iron chelator)	1.9 ± 0.52	2.0 ± 0.42	0.7996	3,4	40	p.o.	qdx11
Vehicle		1.7 ± 0.17	3.0 ± 0.44	0.0061	12,5			

Tumor-bearing MMTV-*Neu* mice were treated with either 1MT (+1MT) or placebo (–1MT) in combination with the cytotoxic drugs and molecular therapeutic agents indicated (at the doses indicated). We scored tumor volumes just before and 2 weeks after initiation of therapy. Fold changes in tumor volumes were determined and the means are presented for each group. i.v., intravenous; i.p., intraperitoneal; p.o., per os (orally); qdx11, once a day for 11 d. *P* values compare +1MT and –1MT groups.

(**Fig. 3a**). Moreover, immune depletion of $CD4^+$ T cells from syngeneic animals abolished the ability of 1MT to suppress *Bin1*$^{-/-}$ tumor growth (**Fig. 3b**). 1MT does not seem to be directly cytotoxic or growth inhibitory, as it did not affect tumor growth in nude or immune-depleted syngeneic mice, nor were cytotoxicity or growth inhibition observed when MRKECs were treated with 1MT *in vitro* (data not shown). We conclude that IDO elevation is a critical mediator of immune escape caused by *Bin1* loss.

IDO inhibitors potentiate cancer chemotherapy

We next investigated whether IDO is critical for tumor survival in MMTV-*Neu* mice, a well-accepted transgenic mouse model of breast cancer, which, like human malignant breast cancers, shows attenuation of Bin1 expression (**Supplementary Fig. 2** online). MMTV-*Neu* mice bearing autochthonous tumors were randomly enrolled into control and treatment groups when tumors reached a diameter of 5–10 mm. 1MT was administered as in the MRKEC tumor graft setting described above. In some trials, we combined 1MT with paclitaxel, a chemotherapeutic agent used for breast cancer treatment, based on reports that taxanes promote T-cell infiltration of tumors[27]. By itself, 1MT retarded but did not arrest outgrowth of autochthonous tumors (**Fig. 4a**). Combinations with either IFN-γ or interleukin (IL)-12 did not accentuate the effect of 1MT (data not shown), arguing that IDO inhibition could not compromise the survival of established tumor cells, even when combined with immune stimulatory cytokines.

In marked contrast, combining 1MT with various cytotoxic agents led to tumor regressions under conditions where single agents were ineffectual. Combining 1MT with paclitaxel resulted, on average, in a 30% decrease in tumor volume within 2 weeks of initiating therapy, whereas paclitaxel delivered near the maximum-tolerated dose (MTD) only slightly retarded tumor growth (**Fig. 4a**). Titrating each agent individually indicated that efficacy was retained at a sub-MTD dose of paclitaxel (4.3 mg/kg administered three times per week) whereas a partial response was observed at a one-quarter dose of 1MT (one 10-mg, 14-d release pellet) (data not shown). Histopathological analysis of tumor sections from 1MT + paclitaxel–treated mice offered evidence of increased tumor-cell death (**Fig. 4b**). Immune depletion of $CD4^+$ T cells limited tumor growth relative to control tumors during the experiment but, as expected, it abolished the ability of 1MT + paclitaxel treatment to elicit tumor regression (**Fig. 4c**). This effect correlated with a reduction in tumor-cell death as evidenced by histological analysis of tumor sections (**Fig. 4d**).

It is unlikely that a drug-drug interaction simply caused an increase in the effective dose of paclitaxel, which was administered near the MTD[28], because we did not observe characteristic neuropathies that would be expected to occur if 1MT had increased the effective dose (data not shown). Furthermore, this interpretation does not explain the effectiveness of combining 1MT with other drugs cleared by different mechanisms. We ruled out the trivial possibility that high doses of a tryptophan-like compound were sufficient by showing that DL-tryptophan was ineffective when substituted for 1MT in the regimen (data not shown). Lastly, we further confirmed the requirement for T cell–dependent immunity by showing in a tumor graft model, using an MMTV-*Neu*–derived tumor cell line, that 1MT showed effects only when the tumors were established in immunocompetent syngenic mice and was ineffectual in T cell–deficient athymic nude mice (**Supplementary Fig. 3** online).

We next evaluated the effects of combining 1MT with other cytotoxic drugs with diverse mechanisms of action that are used to treat breast cancer (**Table 1**). Among the agents tested were the DNA alkylating drugs cisplatin and cyclophosphamide, the topoisomerase inhibitor doxorubicin (adriamycin), the antimetabolites 5-fluorouracil and methotrexate, and the antimitotic agent vinblastine. We also tested several molecular targeted agents, including a farnesyl transferase inhibitor (FTI; L-744,832)[29], the mTOR pathway inhibitor rapamycin, and the angiogenesis inhibitor tetrathiomolybdate. 1MT cooperated

with cisplatin, cyclophosphamide and doxorubicin to elicit mean tumor regressions that differed significantly ($P < 0.05$) from the impact of single-agent therapy (**Table 1**). Mean tumor regression was also produced in combination with FTI; however, as a result of the growth suppression produced by FTI alone, the differential with the combination treatment did not meet the threshold for assigning significance ($P > 0.05$; **Table 1**). We conclude that IDO inhibition cooperates with diverse chemotherapeutic agents to effectively promote regression of established breast tumors that are refractory to chemotherapy.

A new inhibitor of IDO with antitumor activity

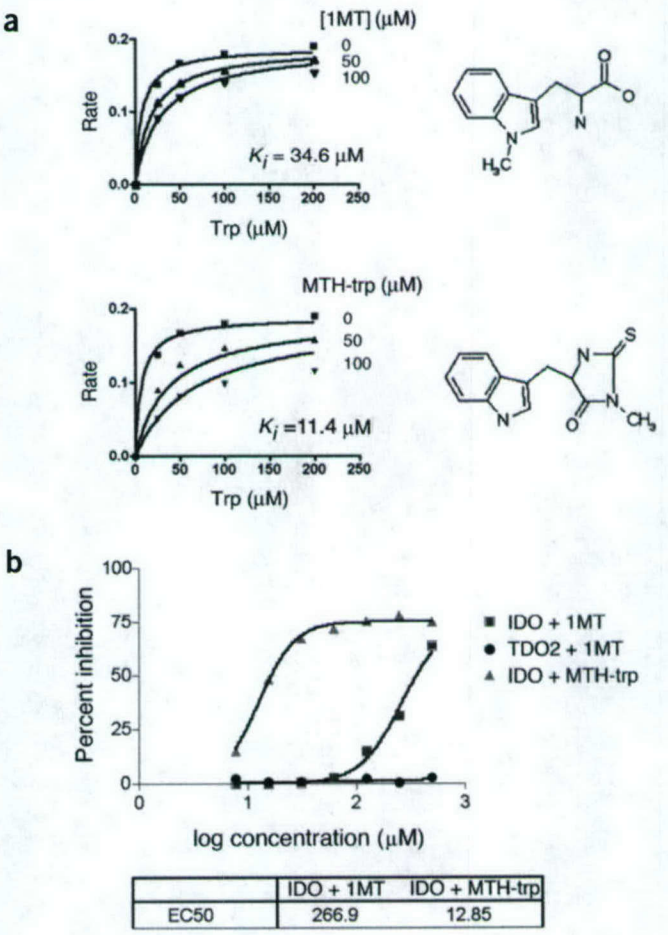
The observations described above are consistent with the presumptive specificity of 1MT for IDO; however, we wished to address off-target concerns by examining the ability of a structurally distinct inhibitor of IDO to elicit tumor regression in combination with paclitaxel. Toward this end, we screened for bioactive inhibitors among commercially available indoleamine-containing compounds, using a purified recombinant human IDO enzyme for *in vitro* assays and a human *INDO* cDNA for expression in cell-based assays. Both assays used a colorimetric method to quantify the production of kyneurinine, the product of reaction catalyzed by IDO[30]. Several compounds were identified that showed inhibition constants against recombinant human IDO that were within about two- to threefold of 1MT ($K_i = 34.2$ μM). One active compound was methyl-thiohydantoin-tryptophan (MTH-trp), which biochemical analyses showed to be a competitive inhibitor with $K_i = 11.6$ μM (**Fig. 5a**). Cell-based screens were performed after transient expression of human *INDO* cDNA in COS-1 monkey cells. As a counterscreen for selectivity, the inhibitory activity of the compounds against the structurally distinct liver enzyme TDO2 was also determined. MTH-trp was ~20-fold more potent than 1MT in the cell-based assay (**Fig. 5b**). Two other thiohydantoin derivatives of tryptophan with lower potency than MTH-trp were also identified (data not shown), confirming the IDO-inhibitory nature of this structural class and suggesting that the thiohydantoin sidechain is probably a mimetic of the amino acid backbone in tryptophan (**Fig. 5a**). Pilot pharmacology experiments have indicated that MTH-trp is more soluble in aqueous solution than 1MT but is also more rapidly cleared from serum; both compounds were found to be orally bioavailable (**Supplementary Fig. 4** online).

Using the same formulation, route of delivery and dose used previously to administer 1MT, we observed that combining MTH-trp with paclitaxel produced regression of tumors as well or better than 1MT (**Fig. 6a**). At the same 2-week endpoint, autochthonous tumors subjected to the combination therapy regressed, on average, 45% relative to the starting tumor volume. One mouse in the trial showed complete tumor regression. Histological examination of tumor sections confirmed evidence of tumor-cell death elicited by the combination therapy, as expected (**Fig. 6b**). Like 1MT, MTH-trp administered by itself retarded tumor outgrowth but did not promote regression. In addition, MTH-trp produced no evidence of gross toxicity in the mice during treatment or at necropsy. Compound titration showed that combinatorial efficacy was fully retained when the MTH-trp dose was reduced by half and partly diminished in response to a one-quarter dose (data not shown). The ability of MTH-trp to effectively combine with paclitaxel in a similar manner as 1MT strengthens the interpretation that the basis for this cooperativity is through IDO inhibition.

DISCUSSION

Immune escape is a central hallmark of cancer, but compared to other recognized hallmarks of cancer—immortalization, suppressor loss, sustained growth, apoptotic resistance, angiogenesis, invasion and metastasis[31]—much less is known about the genetics of immune escape. Here we address this gap in knowledge by defining a genetic mechanism that restricts the ability of cancer cells to escape T cell–dependent antitumor immunity. Using a mouse knockout model, we have shown that *Bin1* can restrain immune escape of oncogenically transformed cells by restricting expression of IDO, an immunoregulatory enzyme that is widely elevated in human cancer[21]. *Bin1* is likewise widely attenuated or mis-spliced in cancers of the breast, prostate, colon, brain and other organs[3,5–8,32,33] (K. Xie, L. Wang, J.B.D. and G.C.P., unpublished data). The finding that IDO is under negative genetic control by *Bin1* offers mechanistic insight into how immune escape may be enabled during cancer progression.

Translational studies prompted by our findings have led us to identify a new strategy for cancer treatment that combines IDO inhibitor–based immunomodulation with cytotoxic chemotherapy. Although gene ablation studies are needed to fully validate IDO as a therapeutic target, the chemical genetics strategy used here offers an initial line of support. Combination drug treatment for cancer is the standard of care, but few studies have explored combinations of immunomodulating agents with chemotherapy. Our findings argue that immunotherapy and chemotherapy can be combined to more effectively destroy cancer cells,



	IDO + 1MT	IDO + MTH-trp
EC50	266.9	12.85

Figure 5 MTH-trp is a potent bioactive inhibitor of IDO. (**a**) *In vitro* enzyme assay. Global nonlinear regression analysis of enzyme kinetic data obtained for human IDO in response to 1MT and MTH-trp. Computed K_i values are shown for each compound. (**b**) Cell-based assay. Results of dose-escalation studies over two logs are shown for 1MT against both IDO and TDO2 and for MTH against IDO. EC_{50} values determined by nonlinear regression are shown.

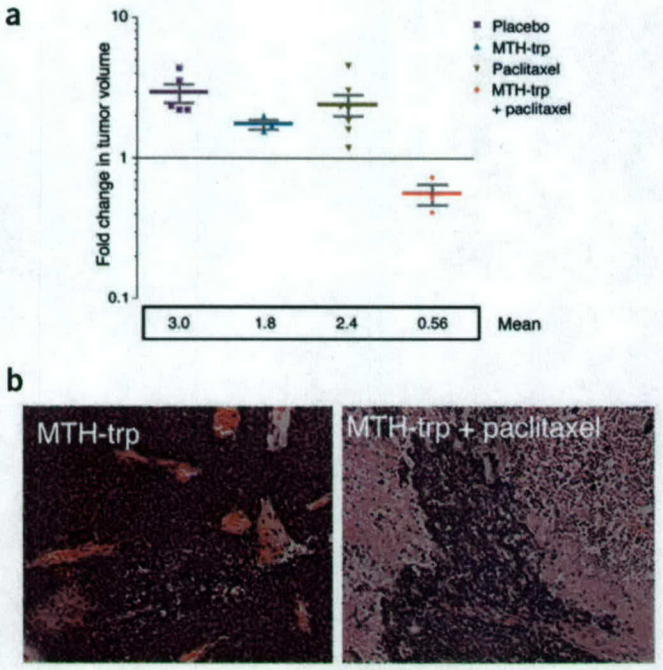


Figure 6 MTH-trp enhances paclitaxel efficacy. (**a**) Therapeutic response. Tumor-bearing mice were implanted with time-release pellets containing MTH-trp (20 mg/d) pellets. Paclitaxel was administered and tumor responses were scored as described in **Fig. 4a**. Results from placebo control and paclitaxel-only treatment groups from **Figure 4** are provided for comparison. Each point represents the fold change in volume for an individual tumor with the mean ± s.e. indicated for each group (n = 5,3,7,4 in the order graphed). The MTH-trp + paclitaxel group includes one complete tumor regression that cannot be plotted on the log scale. (**b**) Tumor histology at endpoint. Representative hematoxylin and eosin–stained sections photographed using a 10x objective on an Olympus BH2 microscope.

consistent with two other preclinical studies that focused on different immunotherapeutic principles[34,35].

Bin1 restrains IDO at the level of IFN-γ-regulated transcription by limiting the induction of IDO message by STAT1 and NF-κB, two key regulators of immunity and cancer. Precisely how *Bin1* influences the STAT1- and NF-κB-dependent transcription of *Indo* remains to be determined. Because nuclear localization is important for the suppressor activity of Bin1 isoforms[4], nuclear actions may be relevant. Because of a lack of mechanistic understanding, most studies of Bin1 adapter proteins have ignored evidence of nuclear localization of the ubiquitous isoforms[3,33,36,37], despite an established precedent for nuclear localization of other 'endocytotic-like' proteins (*e.g.*, epsin and CtBP, also known as BARS[38–40]). Nuclear localization is also intriguing given intrinsic transcriptional repression activity associated with the Bin1 BAR domain[4] (M. Huang, P.S.D. and G.C.P., unpublished data). Indeed, a recent study of the BAR adapter protein APPL reinforces this concept by showing not only its nuclear trafficking function but also its association with the chromatin remodeling complex NuRD/Mi-2 that represses transcription[16]. In summary, effects on trafficking and/or transcriptional repression by *Bin1* may be relevant to its regulation of IDO.

This study extends the evidence that *Bin1* limits cancer pathophysiology and *Myc* oncogenicity[3–9,22,41,42]. Earlier work on cell-intrinsic suppressor roles of *Bin1* are expanded here by the identification of a cell-extrinsic suppressor role that involves restraining a protoleragenic mechanism that tumor cells can exploit to escape antitumor immunity. These cell-intrinsic and extrinsic suppressor roles might be intertwined given the complex involvement of the immune stromal environment in cancer pathophysiology. *Myc* overexpression is associated with major histocompatibility complex (MHC) class I downregulation in neuroblastomas and melanomas[43,44], two cancers in which *Bin1* is often attenuated by mis-splicing[5,8]. Thus, MHC class I downregulation may cooperate with Bin1-IDO dysregulation to facilitate immune escape by cells that overexpress *Myc*. The presence of IFN-γ in the tumor microenvironment may provide a selective pressure to explain the attenuation of *Bin1* and the upregulation of IDO during cancer progression.

This is the first study to link IDO to a cancer suppression pathway. Elevated tryptophan catabolism in cancer patients has been recognized for decades[45,46], a phenomenon that can be explained by IDO overexpression in tumors[21]. IDO catalyzes the initial step in tryptophan catabolism that leads to biosynthesis of nicotinamide adenine dinucleotide. But mammals salvage rather than synthesize nicotinamide adenine dinucleotide, and a different liver-specific enzyme, tryptophan dioygenase, metabolizes dietary tryptophan. Thus, the biological role of IDO was obscure until it was shown that localized tryptophan catabolism forms the basis for a unique mechanism of establishing peripheral tolerance[19]. Because IDO inhibition is not inherently cytotoxic, identification of IDO inhibitors would elude traditional cytotoxic drug screens that are based on tumor-cell survival in tissue culture or in xenograft mouse model systems. In immunocompetent mice, we found that IDO inhibitors potentiated the efficacy of certain cytotoxic drugs without increasing their side effects. The mechanistic basis for the cooperation is not yet clear. Cooperating cytotoxic agents may induce certain types of cell death (*e.g.*, apoptotic versus nonapoptotic death) that elevate presentation of tumor antigens. Alternately, cooperating cytotoxic agents may preferentially compromise the survival of regulatory T cells relative to effector cells, contributing to a weakening of immune tolerance and stimulation of antitumor immunity[27,34,35]. In future work, it will be important to distinguish these possibilities, for example, by determining whether combinatorial efficacy is retained against drug-resistant tumor cells and whether the cytotoxic drugs that are effective in combination with IDO inhibition have similar effects on the immune system, despite their diverse cytotoxic mechanisms.

Our findings are consistent with the interpretation that IDO activity in tumor cells is the relevant target for inhibition; however, they are not incompatible with the possible involvement of stromal APCs, in which IDO can also be highly expressed (*e.g.*, as in the draining lymph nodes of breast tumors[47]). Thus, it is possible that IDO inhibitors may act in part by blocking an immune-tolerizing activity of APCs that are located at distal sites. From a therapeutic standpoint, the possibility that IDO may be a stromal target increases its appeal because of the reduced likelihood of selecting for drug resistance (relative to genetically plastic tumor cells), and because of the increased range of tumors that might be treated even in the absence of direct IDO overexpression. In closing, this study proposes IDO as an attractive and tractable target for the development of small-molecule immunomodulatory drugs to safely leverage the efficacy of standard chemotherapeutic agents.

METHODS

Drugs and chemical compounds. We purchased drugs as clinical formulations or formulated them as described in **Supplementary Methods** online. For *in vitro* studies, 1MT (Sigma) and MTH-trp (Sigma) were formulated in dimethyl sulfoxide (DMSO), including 0.1 N HCl for the less soluble compound 1MT. For *in vivo* studies, 1MT and MTH-trp were formulated in 2-week time-release pellets (Innovative Research).

Tissue culture. We obtained and cultured primary skin keratinocytes from E18.5 days post-coitus mouse embryos on a mixed 129sv/BL6 background[12], essentially as described[47]. Transformed cell populations, referred to as MRKECs, were generated by infection with ecotropic helper-free *Myc* and *Hras1* recombinant retroviruses and analyzed *in vitro* as detailed in **Supplementary Methods** online.

Northern and western blot analyses. We used standard methods as described in **Supplementary Methods** online. We generated a mouse monoclonal antibody recognizing the mouse and human IDO proteins (clone 10.1; UBI) essentially as described[49], using a bacterially expressed peptide encoding amino acids 78–184 of the human IDO protein.

Transcription assays. Cells seeded overnight in 12-well dishes were transfected with 200 ng mIDOprom900-luc, a luciferase reporter plasmid containing 900 base pairs of the mouse *Indo* promoter and 70 nucleotides of noncoding sequences in exon 1, 100 ng CMV-β-galactosidase (to normalize transfection efficiencies) and 700 ng of CMV-Bin1 plasmids as noted. Total DNA in each transfection was made up to 1,000 ng with the analogous CMV empty vector (pcDNA3-neo; Invitrogen). Detailed protocols for transfection and normalized reporter analysis, as well as siRNA sequences, are provided in **Supplementary Methods** online.

Tumor formation and drug response assays. For tumor formation by MRKECs, we injected 1×10^6 cells subcutaneously into syngeneic F1 offspring from 129S1/SvImJ and C57BL/6J breeders (Jackson Laboratories) and into immunocompromised CD-1 Nude (Crl:CD-1-nuBR) mice (Charles River Laboratories). Four weeks after cell injection, mice were killed and tumor mass was determined. We generated autochthonous mammary gland tumors in multiparous female MMTV-*Neu* mice harboring the normal rat *HER2/Neu/ErbB2* gene (Jackson Laboratories). The incidence of detectable tumors in this model is ~80% at 7 months of age and increases to nearly 95% at 8 months. To monitor drug responses, we randomly enrolled tumor-bearing mice into control and experimental treatment groups when tumors reached 0.5–1.0 cm in diameter. Based on the release rate computed by the vendor, the total dose delivered by subcutaneous time-release pellets was at least 20 mg/d, confirmed in pilot tests for a period of up to 5 d by pharmacokinetic analysis of blood serum. Control mice received placebo pellets only. Two days after pellet implantation, we delivered all chemotherapeutic agents except FTI and tetrathiomolybdate by bolus intravenous injection into the tail vein on a schedule of three times per week for a period of 2 weeks. FTI and tetrathiomolybdate were delivered daily by intraperitoneal injection on the same schedule. The doses used for each cytotoxic agent were at or near the MTD reported in the literature. At the 2-week endpoint, we determined tumor volume and wet weight. Tumors were both frozen and fixed and subsequently processed, sectioned and analyzed by standard methods as described in **Supplementary Methods** online. All methods involving mouse use were approved by the Institutional Animal Care and Use Committee of the Lankenau Institute for Medical Research.

Immune cell depletions. Immunocompetent mice used in this study were subjected to cell depletion using standard methods[23,50]. Briefly, for T-cell depletion, we isolated tissue culture supernatants from the rat hybridomas GK1.5 and 2.43 and used them as a source of CD4-specific and CD8-specific monoclonal antibodies. Mice were injected intraperitoneally with 0.5 mg antibody for 3 d consecutively. We monitored splenic T-cell numbers 3 d later and at the experimental endpoint by flow cytometry using FITC-conjugated antibodies. Cell depletion was maintained during both tumor formation and tumor therapy experiments by intraperitoneal injection of 0.5 mg antibodies every 3 d. This strategy depleted >95% of the targeted T-cell subset in all treated mice compared to control mice that were injected with phosphate-buffered saline. For depletion of macrophages and APCs, we injected mice intraperitoneally with 2 mg carrageenan at 6, 3 and 1 d before subcutaneous injection of MRKECs, after which mice were injected one time per week up to the experimental endpoint.

IDO enzyme assays and inhibitor screens. We purified recombinant human his_6-IDO from *E. coli* strain BL21DE3pLys and used it in enzymatic reactions essentially as described[30]. The biochemical and cell-based inhibitor screening assays that were used to identify new IDO inhibitors were performed in a 96-well plate format, as described in detail in **Supplementary Methods** online.

Accession numbers. The GenBank accession numbers for the human IDO protein and the *Mus musculus* strain C57BL/6J chromosome 8 genomic contig, sequences 3011229–3010328 are NP 002155 and NT_039456, respectively.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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IDO In Cancer: Targeting Pathological Immune Tolerance With Small Molecule Inhibitors

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indoleamine 2,3-dioxygenanse, *Indo*, tryptophan, 1-methyl-tryptophan, 1MT, Bin1, chemotherapy, T cell, dendritic cell, DC, macrophage, antigen presenting cell, APC, tumor

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ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is an interferon-γ (IFN-γ) inducible, extrahepatic enzyme that catalyzes the initial and rate limiting step in the degradation of the essential amino acid tryptophan. Elevated tryptophan catabolism mediated by IDO is associated with a wide variety of human cancers and has historically been thought to be a tumoricidal consequence of IFN-γ exposure. Evidence of a physiological requirement for IDO activity in protecting the allogeneic fetus from rejection by the maternal immune system has stimulated a radical shift in thinking about the role of IDO in cancer. Evidence now suggests that tumors can exploit IDO-mediated peripheral tolerance as an important mechanism for immune escape. This review summarizes key studies that implicate IDO as an important mediator of peripheral tolerance as well as the development of a promising new anti-cancer modality that incorporates the use of IDO inhibitors. The second part then focuses on the current state of development of IDO inhibitory compounds as potential pharmaceutical agents.

INTRODUCTION

Tumor interactions with the host immune system are complex and dynamic. Inflammation produces a tumor promoting environment comprised of cytokines, chemokines, and growth factors, activated stroma, and DNA damaging agents [1]. However, host immunity can also have negative consequences for tumors. In particular, the expression of tumor antigens means that cancer cells must evolve mechanisms to escape or subvert anti-tumor immunity in order to successfully progress [2,3]. This process of 'immune editing' whereby immune-mediated destruction of nascent cancer cells provides selective pressure that shapes the immunogenic phenotype of the growing tumor [4], has been clearly demonstrated in mouse tumor models [5]. Studies of human tumors provides further evidence of a microenvironment of immune privilege that protects cancer cells from immune destruction [6-8]. Immune escape is gaining general acceptance as being one of the 'hallmarks of cancer' [9] and an important obstacle to developing immunotherapeutic protocols such as adoptive immunotherapy of *in vitro* activated T cells, which has been only marginally successful despite evidence that the transferred T cells can localize to tumors [10,11]. A promising target for attacking tumoral immune escape, reviewed here, is the enzyme indoleamine 2,3-dioxygenase (IDO).

BODY OF REVIEW

1. IDO, Immune Regulation, and Cancer

1.1 IDO – Background

Elevated tryptophan catabolism, a condition previously associated with microbial infections such as tuberculosis, was observed in patients with bladder cancer in the 1950s [12]. By the 1960s elevated levels of tryptophan catabolites had been documented in the urine of patients with a variety of malignancies including leukemia, Hodgkin's disease, prostate disorders, and breast cancer [13-18]. The hepatic enzyme tryptophan dioxygenase (TDO2; EC 1.13.11.11) was known at the time to carry out the catabolism of dietary tryptophan, having been the first inducible mammalian enzyme to be isolated back in the 1930s [19,20]. TDO2 catalyzes the initial and rate limiting step in the degradation of tryptophan to N-formylkynurenine. However, no increase in TDO2 activity was detected in patients presenting with elevated tryptophan catobolites, implying the activity of second enzyme.

In 1963 the isolation of a non-hepatic tryptophan catabolizing enzyme, D-tryptophan pyrrolase, was first reported [21,22]. Renamed indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.17), this enzyme also converts tryptophan to N-formylkynurenine. However, despite producing the same reaction product as TDO2, IDO is otherwise remarkably dissimilar [23]. IDO is a monomeric 41 kDa enzyme whereas the active TDO2 enzyme is a tetramer totaling 320 kDa in size. The two proteins are antigenically distinct [24] and share no significant amino acid sequence homology (as determined by standard comparative analysis using the NCBI 'BLAST 2 Sequences' online program). IDO has less stringent substrate specificity, cleaving a number of indole containing compounds that are not recognized by TDO2. This is an advantageous consideration in the

development of compounds that will selectively inhibit IDO but not TDO2, since the IDO active site is likely to accommodate a wider spectrum of inhibitory compounds than TDO2. IDO is a heme containing enzyme that utilizes superoxide anion for activity whereas TDO2 does not use superoxide as an oxygen donor. *In vitro*, IDO enzyme reactions are performed substituting ascorbic acid for superoxide. The IDO enzyme also requires methylene blue as a cofactor in the reaction to maintain full activity. *In vivo*, the role of methylene blue is thought to be assumed by either a flavin or tetrahydrobiopterin. The cofactor binding site is distinct from the substrate binding site [25] and may represent an opportunity for the development of distinct classes of non-competitive IDO inhibitors.

Indo is the official designation for the gene encoding the IDO enzyme. In humans, *Indo* is a single copy gene comprised of 10 exons spanning approximately 15 kb which maps to 8p12-p11 [26,27]. The mouse gene, also located on chromosome 8, has a similar genomic organization., There is, however, a good deal of divergence at the primary amino acid sequence level between species, with the human and mouse *Indo* genes sharing only 62.5% identity. The *Indo* gene is found early in evolution with a homologous gene present in the yeast *Saccharomyces cerevisea* (Genbank no. – Z49578). It appears, however, to have undergone functional divergence during the course of the evolution of archaegastropod mollusks (including *Sulculus*, *Nordotis*, *Battilus*, *Omphalius*, and *Chlorostoma*), which express a unique form of myoglobin derived from the primordial *Indo* gene [28]. This abalone myoglobin provides useful structure/function data regarding the mammalian IDO enzyme. In particular a mutation of a conserved histidine, which was determined to be the most likely iron-bound proximal histidine for the abalone myoglobin, has been shown to also be critical for mammalian IDO activity (S Donover, J DuHadaway, AJM, GCP, unpublished results and [29]). Solving the crystal structure of human IDO, (for which

diffraction data at 2.3 angstrom resolution has been collected according to a recent web posting (S Oda, H Sugimoto, T Yoshida, Y Shiro, unpublished results), will be immensely valuable for structure-activity relationship (SAR) -based modeling of inhibitory compound interactions.

The cytokine interferon-γ (IFN-γ) is a major inducer of IDO, especially in antigen presenting cells such as macrophages and dendritic cells (DCs) [30-33]. Transcriptional induction of the *Indo* gene is mediated through the JAK/STAT pathway, in particular JAK1 and STAT1α [34]. STAT1α appears to act to induce *Indo* gene expression both directly through binding of GAS sites within the *Indo* promoter as well as indirectly through induction of IRF-1 which binds the *Indo* promoter at two ISRE sites [34-38]. NFκB also contributes to *Indo* induction [34]. In particular, IFN-γ and TNF-1 (which signals through NFκB) appear to act synergistically to induce expression of IRF-1 through a novel composite binding element for both STAT1α and NFκB in the IRF-1 promoter (termed a GAS/κB element) that combines a GAS element overlapped by a non-consensus site for NF-κB [39]. A possible alternative to the development of inhibitors that block IDO enzyme activity directly might be to develop compounds that block the induction of *Indo* gene expression. Given the number of other targets regulated by IFN-γ signaling, however, it is likely that blocking the entire signaling pathway would have pleiotropic consequences unrelated to IDO inhibition.

1.2 IDO promotes peripheral tolerance

IDO activity is ubiquitously present, albeit at differing levels, in mammalian organs [40,41]. Unlike TDO2, IDO is unresponsive to changes in tryptophan or glucocorticoid levels [40] and is

therefore unlikely to be responsible for metabolic processing of dietary tryptophan uptake. Particularly high basal levels of IDO activity has been associated with the epididymis, the placenta of pregnant females and at other sites of immune privilege [23,42]. Bacterial lipopolysaccharide (LPS) exposure elevates IDO activity in a variety of mouse tissues, most notably colon and lung [40,41,43]. IDO, like TDO2, catalyzes the initial and rate limiting step in the *de novo* biosynthesis of the critical coenzyme nicotinamide adenine dinucleotide (NAD). However, mammalian cells appear to predominantly depend upon salvage pathways for maintaining NAD pools. Most mammalian tissues outside of liver and kidney lack essential enzymes downstream of IDO for *de novo* NAD biosynthesis and catabolites of tryptophan breakdown by IDO appear primarily excreted in urine [41]. The pattern of IDO expression suggested early on that it was a component of the inflammatory response system [44] though its role remained enigmatic. Accumulating experimental evidence supported the hypothesis that IDO might protect the host from auxotrophic pathogens by depleting the local tryptophan pool and/or the production of toxic catabolites [45,46]. After IDO activity was found to be elevated in various cancer patients, the hypothesis was expanded to include the idea of IDO upregulation being a tumoricidal consequence of IFN-γ exposure through both starvation of the proliferating tumor cells of the essential amino acid tryptophan as well as exposure to cytotoxic tryptophan catabolites [47-51].

In 1998 Munn, Mellor and colleagues published the seminal finding that IDO activity is essential for protecting the allogeneic fetus from the maternal immune system [52]. This study was performed based, in part, on *in vitro* findings indicating that T cells are exquisitely sensitive to tryptophan depletion by macrophages. Upon encountering an activation signal in a low tryptophan environment, T cells were found to be unable complete progression through the cell

cycle, arresting in mid-G1. If not activated again in the presence of sufficient tryptophan, these T cells subsequently underwent apoptosis [53]. The demonstration that macrophages are imbued with the ability to suppress T cell proliferation when differentiated *in vitro* through exposure to macrophage colony-stimulating factor (MCSF) [54,55] had indicated that this might be a useful experimental system to study the mechanistic basis for the establishment of peripheral tolerance. Tryptophan depletion mediated by increased IDO activity was shown to be upregulated in MCSF-differentiated macrophages responding to IFN-γ stimulation [53]. In this *in vitro* study, the small molecule IDO inhibitor 1-methyl-DL-tryptophan (1MT) was used to block macrophage IDO activity. 1MT had been identified as the most effective inhibitor among a small series of tryptophan analogs evaluated for IDO inhibitory activity[56]. A detailed review of the literature on small molecule inhibitors of IDO can be found in Section 2. 1MT then became the key tool employed *in vivo* to demonstrate the essential role of IDO in protecting the developing fetus from maternal immunity[53]. 1MT was delivered by subcutaneous implantation of time-release pellets, which permitted continuous dosing to be achieved. An important caveat with regard to this study as well as all subsequent studies employing 1MT that have been published to date is that none has actually demonstrated that the intended target IDO is inhibited *in vivo* at the dose level delivered. Nor has there been any reported attempt to correlate biological effects of 1MT with IDO dose response *in vivo* by a pharmacodynamic assay. Therefore, the possibility that 1MT may be acting through an off-target mechanism of action has yet to be rigorously addressed.

In another consequential finding, IDO has been implicated in the suppression of T cell activation by CTLA-4 [57]. CTLA-4 is an important mediator of peripheral immune tolerance, and mice that are genetically deficient for CTLA-4 develop fatal autoimmune disease [58,59]. CTLA-4

belongs to the CD28 family of proteins. CD28 is an important costimulatory molecule for activation of T cells through engagement of the T cell receptor (TCR). Both CD28 and CTLA-4, which are expressed on the surface of T cells, bind the B7 ligands, B7-1 and B7-2, expressed on the surface of APCs. The soluble fusion protein CTLA-4-immunoglobulin (CTLA4-Ig), which also binds B7-1 and B7-2, can prevent allograft and xenograft rejection in mouse transplantation models [60-62]. It has generally been accepted that CTLA-4 is directly antagonistic to CD28 in T cells, either through out-competing CD28 for access to B7 ligand, inducing immunosuppressive cytokines, or directly interfering with CD28-mediated and/or TCR-mediated signaling [63]. The first evidence that IDO might be an important mediator of CTLA-4-Ig induced tolerance was the observation that, in a diabetic mouse model, the ability of CTLA-4-Ig to effectively suppress immune rejection of pancreatic islet allografts was lost if IDO activity was concurrently inhibited by treatment with 1MT [57]. The study by Grohmann et al. further suggested that CTLA-4-Ig mediated tolerance occurs through a heterodox mechanism of 'reverse' signaling through B7 molecules on APCs, which promotes IFN-γ production to induce IDO. Subsequent studies have provided further support for and refinement of this model [64-69], which is consistent with CTLA-4 expression at the maternal-fetal interface during gestation [70].

1.3 IDO in anti-tumor immune escape

The concept that IDO activity is physiologically important for establishing peripheral tolerance to paternal alloantigens expressed by the fetus has engendered a complete rethinking of the implications of the elevated IDO activity observed in cancer patients. Induction of IDO was generally thought to be a turmoricidal consequence of IFN-γ exposure as the growing tumor cells

were starved of an essential amino acid as well as exposed to toxic products of tryptophan degradation [48,71-73]. However, cancer cells are highly adaptive, compensating for a low tryptophan environment, for instance, by upregulating tryptophan tRNA synthetase induction in response to IFN-γ [74]. If IDO can block immune responses to the highly antigenic paternal alloantigens expressed by the fetus, it should also be capable of blocking responses to much weaker tumor antigens. Therefore, tumors that can survive the deleterious consequences of IDO upregulation may benefit from its immune suppressive activity. The idea that IFN-γ exposure can have diametrically opposed consequences for tumors is already well established in the literature. For instance, IFN-γ has been shown to cooperate with lymphocytes to protect against the development of both spontaneous as well as chemically-induced tumors, but the tumors that do grow out in this context are more aggressive when transplanted in a syngeneic, immunocompetent host [5]. This is consistent with positive selection for reduced immunogenicity, a phenomenon that has been termed 'immune editing' [4].

Does the relevant upregulation of IDO activity occur in the tumor cells themselves or in the adjacent stroma? Two competing, although not necessarily mutually exclusive explanations have developed regarding this question. Experimental evidence supports the idea that cancer cells with active IDO enzyme can promote immune suppression. A fibrosarcoma cell line ectopically expressing IDO has been shown to directly to inhibit T cell responses [75]. In a separate study, ectopic expression of IDO in a mastocytoma cell line promoted tumor formation in mice that should otherwise have been rendered resistant because of preimmunization [76]. Coupled with the reports of high IDO expression in many tumor-derived cell lines [20] as well as in a high proportion of primary tumor cells from a wide range of tissues [76], it appears likely that direct expression of IDO in tumors can contribute significantly to immune escape.

This raises the question of how IDO becomes dysregulated in tumor cells. One possible answer to this question has come from our own studies of the Bin1 cancer suppression gene. Loss or attenuation of normal Bin1 protein expression during malignant progression has been described in a variety different human tumors including breast cancer, prostate cancer, melanoma, neuroblastoma and colon cancer [77-80] (K Xie, L Wang, JD, GCP unpublished results). To study the mechanistic basis for the apparent selective pressure against Bin1, we used a combination of Myc and Ras oncogenes to transform primary epithelial skin cells (keratinocytes) obtained from Bin1-deficient neonates as well as heterozygous control littermates. The growth characteristics of these transformed cells were virtually indistinguishable *in vitro*, however, when transplanted subcutaneously into syngeneic animals, the Bin1-null cells were aggressively tumorigenic while the Bin1-expressing cells were not. This differential was lost when the cells were injected into athymic nude mice. Prompted by reports that Bin1 could impact STAT and NFκB signaling pathways, we determined that Bin1 is involved in the regulatory control of IDO. IFN-γ mediated induction of IDO is enhanced in Bin1-null keratinocytes and in Bin1-null macrophages as well. As anticipated, treatment with the IDO inhibitor 1MT significantly suppressed the outgrowth Bin1-null MR-transformed keratinocyte tumors in syngeneic animals but had no significant impact on their outgrowth in athymic nude mice. These data provide further support for the conclusion that direct expression of IDO in tumor cells is capable of promoting immune escape and indicate that loss of Bin1 is one mechanism through which IDO dysregulation may occur.

On the other hand, IDO has been implicated in immune escape by tumors that show no direct evidence of IDO expression. It has been argued that this may even be the more relevant means of establishing tolerance [81], though the details on how this might be achieved are still sketchy.

Tumors formed by Lewis lung carcinoma cell line, which did not directly express detectable IDO, induced upregulation of IDO expression in the draining lymph nodes and treatment of mice with the IDO inhibitor 1MT delayed the outgrowth of these tumors [82]. The stromal cells most likely to be providing the IDO activity in this scenario are APCs such as macrophages and dendritic cells. Not all APCs appear to induce IDO, however, and a number of cell surface markers that may help characterize particular APC subsets that are key to mediating this immune regulatory mechanism have been reported [64,67,83-85]. In particular, a plasmacytoid class of dendritic cells identified in mice, which express B cell surface markers and may originate from the B cell lineage, appear to be important expressors of IDO [84,85]. Speculative mechanisms for how tumors induce IDO in proximal APCs are suggested by experimental systems in which evidence for IDO mediated tolerance has been demonstrated. As described previously, CTLA-4 co-receptor has been implicated in the induction of IDO in APCs through B7 ligation. CTLA-4 is highly expressed on regulatory T cells (Tregs) which have been implicated in mediating IDO induction in the DC population [66]. Treg recruitment or generation at the tumor site might thus be a mechanism for cancer cells to indirectly promote local upregulation of IDO activity. Another co-receptor 4-1BB has recently been implicated in the promotion of tolerance in the collagen induced arthritis (CIA) model in mice [86]. In this case, ligation of 4-1BB with an antibody stimulates the accumulation of CD11b+ CD8+ cells that produce high levels of IFN-γ. This induces IDO in responsive APCs which leads to tolerization. Interestingly, aberrant expression of 4-1BB ligand has been reported in solid tumors [87,88], suggesting the possibility that tumors that do not directly express IDO might signal through 4-1BB as an alternative mechanism to induce local IDO activity.

Divergent opinions exist as well as to the mechanism by which IDO promotes immune suppression, namely whether this is due to the local depletion of tryptophan levels or the local accumulation of toxic tryptophan catabolites. Of course these two possibilities are not necessarily mutually exclusive. *In vitro* data favoring each model has been reported. Shortly after publishing the fetal protection study, Munn, Mellor and colleagues published an *in vitro* study that demonstrated that T cells are exquisitely sensitive to tryptophan levels during activation [53]. Low tryptophan levels in the media promoted cell cycle arrest and eventually apoptosis if a subsequent activating signal in the presence of sufficient tryptophan was not encountered. These and subsequent experiments formed the basis for the tryptophan depletion model and provided arguments against relevance of metabolic products [89]. Evidence reported from other laboratories, however, that tryptophan catabolites are primarily responsible for suppressing T cell activation [90-92], has bolstered the counter argument that tryptophan depletion by IDO is unlikely to be able to account for the observed biology [93]. It is apparent that *in vitro* systems are too malleable to convincingly resolve this issue and that clear and definitive *in vivo* experiments will be required.

1.4 Targeting IDO as a therapeutic strategy for cancer treatment

The idea that IDO activity might protect tumors from the host immune system clearly suggests that IDO inhibitors might have utility as anti-cancer agents. Given that 1MT is known to be biological active in defeating immunological tolerance of allogeneic concepti, it has clearly been the compound of choice to perform pilot studies. Our own work and that of other's has shown that 1MT exhibits some efficacy as a monotherapy. In tumor growth inhibition studies

(treatment initiated prior to or concurrent with tumor challenge [94]) involving tumor models that either directly or indirectly utilize IDO for immune escape, 1MT treatment did cause significant tumor growth delays but failed to block establishment [76,82]. In a more stringent type of study in which treatment was initiated on established tumors, inhibition of tumor growth with 1MT treatment alone was also observed, however, regression of tumors, a critical preclinical criterion, was not achieved [76,95]. These findings suggest that IDO inhibitor-based, single-agent immunotherapy may have only limited anti-tumor activity. Pilot experiments performed in our laboratory combining 1MT treatment with injection of IFN-γ or IL-12 did not achieve any stronger effects than 1MT alone (AJM, J DuHadaway, GCP, unpublished results). It is not particularly surprising that the response of tumors is less dramatic than that of allogeic concepti, since the tumor antigens that they express are substantially less antigenic than alloantigens and the tumors may be more flexible in employing alternative mechanisms to protect themselves from immune responses as well.

We have further explored this issue in a well-accepted mouse model of breast cancer, the MMTV- *Neu* 'oncomouse', in which formation of mammary gland adenocarcinomas is driven by overexpression of the HER2/ErbB2/Neu proto-oncogene and closely resembles human ductal carcinoma in situ (DCIS) [96]. In particular, we have probed the anti-tumor effects of the IDO inhibitor 1MT in combination with cytotoxic drugs. Such drugs might appear to be a counterintuitive choice, since they can kill the immune cells that IDO inhibitors are supposed to activate. However, we performed these combinations prompted in part by reports that some cytotoxic drugs can promote tumor immune infiltration and anti-tumor responses (e.g. taxanes and others used for breast cancer therapy [97,98]). Paclitaxel by itself produced only growth inhibition of MMTV-*Neu* tumors consistent with published evidence that Neu overexpression in

breast cancer cells confers paclitaxel resistance [99]. 1MT was delivered to tumor-bearing MMTV-*Neu* mice by subcutaneous introduction of time-release pellets: the same delivery route that achieves sufficient 1MT exposure for rejection of allogenic concepti. In contrast to results obtained with 1MT monotherapy, treatment of tumor-bearing MMTV- *Neu* mice with a combination of 1MT + paclitaxel resulted in the regression of tumors [95].

Control experiments in which a pellets were infused with D,L-tryptophan (analogous to the D,L racemic mixture of the 1MT used) did not replicate the observed cooperative effect of 1MT. Thus, the observed effect could not be trivially ascribed to a nonspecific toxicity caused by high dose of the tryptophan-like indoleamine. We did not rule out the possibility of a pharmacokinetic effect of 1MT on paclitaxel, which might increase its effective dose in the mouse. However, this explanation seemed unlikely, because we did not observe evidence of neuropathy (e.g. hind leg dragging) that would be produced in mice by a higher effective dose of paclitaxel. Immune depletion experiments as well as grafting experiments in nude mice confirmed that T cell-mediated immunity is essential for the combination therapy to elicit tumor regression. Similar cooperativity was observed with other chemotherapeutic agents tested but was not universal. The DNA damaging agents cisplatin, cyclophosphamide, and doxorubicin exhibited cooperativity but the anti-metabolites 5-fluorouracil and methotrexate did not. Interestingly, the other mitotic inhibitor tested, vinblastine, did not show cooperativity. Overt toxicity was not evident in any of these trials. Doxorubicin itself produced tumor regression in the MMTV-*Neu* model at higher doses but this was associated with severe side-effects (slumping and inactivity of treated mice). At a lower dose of doxorubicin, 1MT enhanced regression without increasing evident toxicity. Of two signal transduction inhibitors tested, the farnesyltransferase inhibitor showed some evidence of cooperativity whereas rapamycin did not. This, however, may be consistent with

accumulating evidence that anti-cancer effects of FTIs are not be mediated through inhibition of ras signaling and that a DNA damage mechanism may be an alternative explanation[100]. As expected, the iron chelator tetrathiomolybdate, which has been reported to block angiogenesis, also showed no cooperativity with 1MT. The implications of these findings are striking, as they suggest, perhaps counterintuitively, that modulating immunity with small molecule inhibitors of IDO in conjunction with conventional cytotoxic chemotherapeutic drug based treatments might have clinical relevance. This notion that immunotherapy and chemotherapy can be effectively combined to destroy cancer cells is consistent with two other preclinical studies that focused on different immunotherapeutic principles [97,101].

2. IDO Inhibitors: Chemistry and Pharmacology

2.1 Structural Classes of IDO Inhibitory Molecules

There exists only a small collection of reports describing inhibition studies of indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.17). Not surprisingly the studies have focused primarily on derivatives of tryptophan (Trp) and structurally related heterocycles like β-carboline, despite the reported [23,102,103] promiscuity of IDO compared to the related tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11). Both competitive and noncompetitive inhibitors of IDO have been identified. To date, competitive inhibitors are primarily derivatives of Trp, while noncompetitive inhibitors are derivatives of β-carboline.

2.1.1 Competitive Inhibitors

Substrate inhibition with high concentrations (>0.2 mM) of L-Trp was reported [104,105] during early enzymological studies, therefore it is not surprising that Trp derivatives have been

extensively studied. Derivatization of the Trp structure has occurred in three areas: substitution of the indole ring, modification of the amino acid side chain, and modifications of the indole ring.

2.1.1 Tryptophan Indole Ring Substition

Substition of the indole ring of Trp has afforded the most commonly used inhibitor of IDO: 1-methyl-Trp (**1**) [56]. A racemic mixture was originally used by Munn and co-workers in their seminal study of the fetal survival paradox, [52] but subsequent studies [106] have revealed IDO's preference for the natural L (S) isomer of **1** (the more precise Cahn-Ingold-Prelog system of configurational assignment will subsequently be used in preference to the historic D,L system). Stereochemical preference for the natural isomer was also seen with the 6-nitro derivative **18** (Table 1).

Table 1 comprehensively summarizes the range of substituents that have been tested on the indole ring of Trp. The seven most potent compounds based on the reported inhibition data are the five monosubstituted derivatives, 1-methyl (**1**), 5-bromo (**9**), 6-fluoro (**17**), 6-nitro (**18,** S isomer), 7-fluoro (**20**), and the two difluorinated derivatives, 4,7-difluoro (**8**) and 5,7-difluoro (**15**). Excluding the 1-methyl derivative, the six other are electron withdrawing groups [107-109]. Since the three proposed mechanisms[110,111] for IDO catalysis of the conversion of Trp to N-formyl-kynurenine all begin with nucleophilic attack of the pyrrole ring of Trp, electron withdrawing groups on the indole ring would make this step less favorable and slower. Nevertheless, the activity data in Table 1 indicates that the 5-bromo (**9**) and the 6-fluoro (**17**) derivatives still undergo oxidation, therefore some of these compounds still behave as substrates despite their deactivating substitution.

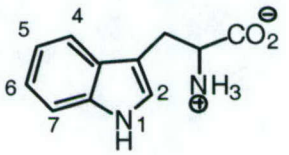
Several compounds, notably the 5-bromo (**9**) and 2-hydroxy (**6**) derivatives, have significantly different values reported for their inhibition. Some of the difference may be due to the different IDO sources and different assay conditions used for the studies. Peterson and co-workers extracted IDO from human monocyte/macrophage cells induced by interferon-γ [106]. They monitored IDO activity by detecting kynurenine product with a radioimmunoassay or HPLC assay. Southan and co-workers used recombinant human IDO purified *E. coli* [112]. They followed IDO activity with a spectrophotometric assay that detected an imine derivative of kynurenine. Several inhibitors reported in later tables isolated IDO from small rabbit intestine and used two different detection methods [56,113]. Nonetheless, despite these differences, several compounds show striking consistency, i.e. **1** and **13** (Table 1) and **39** (Table 2).

Several electron releasing substituents in Table 1 are very active as substrates and are oxidized by IDO: 4-methyl (**7**), 5-methyl (**10**), 5-methoxy (**11**), 5-hydroxy (**13**), and 6-methyl (**16**). One derivative (5-methyl, **10**) is more active than L-Trp. This result is consistent with the mechanistic rational and the outcome described for the electron withdrawing substituents. Electron releasing substituents would be expected to make the indole ring more nucleophilic leading to a faster intial reaction with the oxygen species at the active site.

The 1-methyl derivative **1** defies the trend seen with substituents on the benzene portion of the indole ring. The proposed mechanisms [110,111] for IDO involving pyrrole electron donation, actually initiate the nucleophilic attack with deprotonation of the N-1 hydrogen of Trp. Without a hydrogen, 1-methyl Trp prevents the deprotonation from occurring. Similar inhibition is seen

with benzofuran (**48**, Table 3) and benzothiophene (**49**) analogs of Trp (vide infra). However, there is a limited amount of space in the active site to accommodate N-1 groups as the 1-ethyl (**2**) and 1-phenylsulfonyl (**3**) derivatives exhibited only weak inhibitory activity.

Indole ring substitution of Trp derivatives has been extensively explored; nevertheless the use of multiple substituents is a strategy that might yield more active inhibitors. Excluding compounds **3**, **8** and **15**, there are few compounds with multiplte substituents that have been synthesized and tested. The synthetic challenge posed by polysubstituted indoles is probably one reason that these examples are limited. Another limitation would appear to be the space available in the indole binding region of the active site as seen in the weak activity and inhibition with **12**. Despite these limitations, it is clear that a range of substituents has been accommodated and therefore combinations of these might afford synergistic inhibition. Unlike the β-carboline derivatives (vide infra), there has been no indication of slow-binding inhibition from Trp derivatives; the pre-incubation inhibitory data in Tables 1-3 does not substantially differ from the per cent inhibition found in standard competition assays.



Compound	Indole Ring Substition	Stereochemistry at α position	Inhibition Data (%)[a]	Activity data (%)[b]	Reference
1	1-CH_3	S (L)	52.3 (62.9)[c]; K_i=34 μM[c]		[106]
1	1-CH_3	R,S	26; K_i=6.6 μM[d]	7	[112]
1	1-CH_3	R (D)	5.7 (11.6)[c]		[106]
2	1-CH_2CH_3	S	13.5 (9.9)[c]		[106]
3	1-SO_2Ph, 6-OCH_3	R	3.2 (28.4)[c]		[106]
4	2-Cl	S	20	33	[112]
5	2-Br	S	11	21	[112]

6	2-OH	S	30	4	[112]
6	2-OH	R,S	-38.4 (-43.3)[c]		[106]
7	4-CH_3	R,S	26	33	[112]
8	4-F, 7-F	S	K_i=40 μM		[110]
9	5-Br	R,S	0[c]		[106]
9	5-Br	R,S	56	36	[112]
10	5-CH_3	R,S	6	123	[112]
11	5-OCH_3	R,S	35	70	[112]
12	5-OCH_2Ph	R,S	2	1	[112]
13	5-OH	S	12	59	[112]
13	5-OH	S	14[c]		[106]
14	5-F	R,S	32	46	[112]
15	5-F, 7-F	S	K_i=24 μM		[110]
16	6-CH_3	R,S	20	72	[112]
17	6-F	R,S	54	38	[112]
18	6-NO_2	S	52	2	[112]
18	6-NO_2	R	7	0	[112]
19	7-CH_3	R,S	36	18	[112]
20	7-F	S	K_i=37 μM		[110]

[a] Unless otherwise stated, inhibition data is reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 1 mM of inhibitor. Percent in parenthesis lists inhibition data with 2 h pre-incubation of inhibitor with IDO.

[b]Percent compound oxidized relative to L-tryptophan.

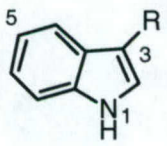
[c]100 μM inhibitor concentration used in inhibition assay.

[d]K_i determined at pH 8.0 in reference 2.

Table 1: Trp Derivatives with Indole Ring Substitution

2.1.2 Tryptophan Side Chain Modifications

A range of Trp side chain modifications have been explored as illustrated in Table 2. However, relatively few have afforded compounds with promising inhibition. Modest inhibition was realized with the addition of a methyl group to either the α-amine (**27**) or the α-acid (**29**). One notable derivative with interesting activity and a novel structure is the thiohydantoin derivative **40** [95]. Further modification of the thiohydantoin ring might provide even more potent inhibitors.



Compound	R=[a]	Stereo-chemistry at α position	Inhibition Data (%)[b]	Activity data (%)[c]	Reference
21	$-CH_2CH_2NH_2$		28	32	[112]
22	$-CH_2CH_2NH_2$; {5-OCH_3}		-43.9[d]		[106]
23	$-CH_2CH_2NH_2$; {2-CO_2H}		16.3 (17.9)[d]		[106]
24	$-CH_2CH_2NH_2$; {2-CO_2H , 5-OCH_3}		10.8 (3.4)[d]		[106]
25	$-CH_2CH_2CO_2H$		0	8	[112]
26	$-CH_2C(CH_3)(NH_2)CO_2H$	R,S	1	35	[112]
27	$-CH_2CH(NHCH_3)CO_2H$	S	33	21	[112]
28	$-CH_2CH(NHCOCH_3)CO_2H$	S	7	3	[112]
29	$-CH_2CH(NH_2)CO_2CH_3$	S	30	15	[112]
30	$-CH_2CH(NH_2)CO_2CH_2CH_3$	S	7	14	[112]
31	$-CH_2CH(OH)CO_2H$	R,S	9.7 (1.4)[d]		[106]
32	$-CH_2N(CH_3)_2$		-6.6[d]		[106]
33	$-CH_2CN$		3.5[d]		[106]
34	$-C(O)NH_2$; {5-OH}		0[d]		[106]
35	-CHO		4.4[d]		[106]
36	$-CH=CHCO_2H$		2.5 (3.2)[d]		[106]
37	$-CH=CHCO_2CH(CH_3)_2$		15.2 (11.6)[d]		[106]
38	-(E)-CH=CH-(3-pyridinyl); {6-F}		0		[114]
39	$-CH(CH_3)CH(NH_2)CO_2H$	α-S,β-S; α-R, β-R	0.0 (-2.7)[d]		[106]
39	$-CH(CH_3)CH(NH_2)CO_2H$	α-S,β-R; α-R, β-S	9.8 (3.6)[d]		[106]
39	$-CH(CH_3)CH(NH_2)CO_2H$	R,S	7	32	[112]
40	$-CH_2$-5'-(3'-methyl-2'-thioxo-4'-imidazolinone)	R,S	K_i=11.4 μM		[95]
41	$-CH_2CH(NH_2)CO$-(S)-Trp	S	K_i=147 μM		[106]

[a] Additional indole substituents are added in brackets.

[b] Unless otherwise stated, inhibition data is reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 1 mM of inhibitor. Percent in parenthesis lists inhibition data with 2 h pre-incubation of inhibitor with IDO.

[c] Percent compound oxidized relative to L-tryptophan.

[d] 100 μM inhibitor concentration used in inhibition assay.

Table 2: Trp Side Chain Modifications

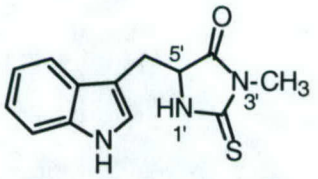
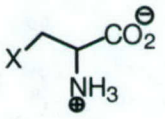


Figure 1: Compound **40**

2.1.3 Indole Ring Modifications

Modifications of the indole ring have afforded a few novel competitive inhibitors (Table 3). Most notable amongst this group are the benzofuran (**48**) and benzothiophene (**49**) derivatives described earlier. These two compounds, like 1-methyl-Trp (**1**), lack an N-1 proton and therefore can not be deprotonated; the initial step in the proposed catalytic mechanism of IDO indole oxidation [110,111]. Attempts at identifying feedback inhibition from subsequent intermediates in the kynurenine pathway failed with the kynurenine analog **46** and the 3-hydroxykynurenine analog **47**. Surprisingly, based on the success of electron withdrawing groups on the benzene portion of the indole (Table 1), a π deficient analog of indole, 7-azaindole (**43**), also failed to demonstrate inhibitory activity. Similarly, modifications of the pyrrole portion of the indole ring, i.e. reduction (**44**) or incorporation of another nitrogen (**42**), also failed to afford inhibition. The majority of the data from Table 3 indicates that the indole ring is almost essential for the creation of a competitive inhibitor.



Compound	X=	Stereo-chemistry at α position	Inhibition Data (%)[a]	Reference
42	3-(1H-indazolyl)-	R,S	0.0	[106]
43	3-(7-azaindolyl)-	R,S	-1.6	[106]

44	3-indolinyl	S	0.4 (3.0)	[106]
44	3-indolinyl	R	-2.4 (-1.2)	[106]
45	3-quinolinyl	S	0	[106]
45	3-quinolinyl	R	0	[106]
46	(2-amino-phenyl)methyl	S	-0.3	[106]
47	(2-amino-3-hydroxy-phenyl)methyl	R,S	-0.4	[106]
48	3-benzofuranyl	R,S	43[b,c]	[112]
48	3-benzofuranyl	R,S	K_i=25 μM	[104,105]
49	3-benzothiophenyl	R,S	16[b,d]	[112]
49	3-benzothiophenyl	R,S	K_i=70 μM	[104,105]
50	1-(1,4-cyclohexadienyl)	S	K_i=230 μM	[115]

[a] Unless otherwise stated, inhibition data is reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Percent in parenthesis lists inhibition data with 2 h pre-incubation of inhibitor with IDO.
[b] 1 mM inhibitor concentration used in inhibition assay.
[c] Twenty-two percent of **44** was oxidized by IDO.
[d] Nineteen percent of **45** was oxidized by IDO.

Table 3: Indole Ring Modifications of Trp

2.1.4 Miscellaneous Structures

A small selection (Table 4) of structures unrelated to Trp has been tested for competitive inhibition. Similar to the modified indole ring structures in Table 3, the majority of the structures have not shown any inhibitory activity. Feedback inhibition was not detected with kynurenic acid (**54**) or quinolinic acid (**57**), nor was inhibition seen with the structurally related analogs **53**, **55** and **56**. Two interesting exceptions were discovered with **52** and **58**. 3-Amino-2-naphthoic acid (**52**) is an analog of anthranilic acid, an intermediate in the aromatic pathway of Trp metabolism. Although assay differences preclude direct comparisons of the potency of IDO inhibitors, compound **52** is one of the most potent inhibitors yet reported in the literature. It is clearly one of the most interesting lead compounds, notwithstanding the synthetic challenge of constructing 3-amino-2-naphthoic acid analogs. A second unique inhibitor was pyrrolidine dithiocarbamate (**58**)[116]. This anti-oxidant demonstrated notable inhibitory activity of IDO generated from interferon-γ treatment of human monocyte-derived macrophages. It is possible

that the sulfur of the dithiocarbamate is binding to the heme iron in the active site of IDO. This binding mode would be consistent with sulfur's well-known affinity for iron in biological systems, e.g. ferrodoxin.

Compound	Structure	Inhibition Data (%)[a]	Reference
51	1-amino-2-naphthoic acid	-2.0 (11.2)	[106]
52	3-amino-2-naphthoic acid	74.2 (75.2)	[106]
53	3-quinolinecarboxylic acid	-2.6	[106]
54	4-hydroxy-2-quinolinecarboxylic acid	1.1	[106]
55	4,8-dihydroxy-2-quinolinecarboxylic acid	2.9	[106]
56	2-picolinic acid	1.5	[106]
57	quinolinic acid	6.8	[106]
58	pyrrolidine dithiocarbamate	44[b]; IC_{50}=6.5-12.5 μM	[116]

a Unless otherwise stated, inhibition data is reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Percent in parenthesis lists inhibition data with 2 h pre-incubation of inhibitor with IDO.
b 125 mM inhibitor concentration used in inhibition assay.

Table 4: Other Compounds Tested for Competitive Inhibition

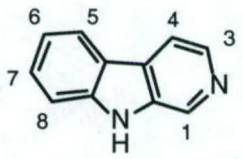
2.1.5 Noncompetitive Inhibitors

The first class of structures exhibiting IDO inhibition was a series of β-carboline structures reported in 1984 [113]. Initially, they were reported to exhibit uncompetitive inhibition, but β-carboline (**59**), also known as norharman, was subsequently reclassified as a noncompetitive inhibitor [117]. β-Carboline derivatives (Table 5) continue to be the most common type of non-competitive inhibitor, but there are also three novel structures (Table 6) that have also been reported[118].

2.1.6 β-Carboline Derivatives

Modifications to the β-carboline structure have occurred in both the pyridine and the benzene rings. The pyridine ring has been reduced and substituted at C-1 and C-3. The benzene ring has been substituted at C-6 and C-7. There are still many positions of the β-carboline structure that have not been explored. The most potent IDO inhibitors have larger alkyl substituents in the C-3 position, e.g. **74** and **76**. There appears to be a hydrophobic pocket in the active site capable of accommodating these alkyl groups. Fluorine and the isothiocyanate group were present in several potent C-6 substituted β-carboline derivatives, e.g. **68**, **76**, and **77**.

As noncompetitive inhibitors, β-carboline derivatives do not compete for the same active site location as Trp or other indoleamine substrates. Nevertheless, there is experimental evidence that indicates that β-carboline **59** binds directly to the heme iron at the active site as a nitrogen ligand and competes with oxygen for binding at the active site iron[117]. Sono has determined that the β-carboline occupies another binding site close to the L-Trp binding region and he hypothesizes that this space may be available for a natural co-factor or a regulator of the enzyme[25]. Interestingly, several of the β-carboline inhibitors (i.e. **61**, **64**, and **66**) demonstrated considerably greater potency on pre-incubation with IDO. This is indicative of slow-binding inhibition and may indicate these inhibitors need time to settle into the second binding pocket near the heme iron. One important liability of β-carboline derivatives is the reported neuroactivity of these structures as benzodiazepine receptor ligands [119-122]. Although many previous IDO inhibitor studies were developing neurotherapies, neuroactivity would be a problematic side effect of a cancer therapy.



Compound	β-Carboline Ring Substitution	Inhibition Data (%)[a]	Reference
59	none	50.3 (57.0); K_i=178 μM	[118]
60	3-OCH_2CH_3	5.5 (21.2)	[118]
61	3-$OCH_2CH_2CH_3$	16.7 (76.7) ; K_i=98.0 μM	[118]
62	3- OCH_2CH_2OH	6.7 (11.0)	[118]
63	3-CO_2t-Bu	7.0 (7.2)	[118]
64	3-$C(O)CH_2CH_2CH_3$	-4.1 (44.9)	[118]
65	3-NH_2	0.9 (-19.4)	[118]
66	3-N=C=S	26.7 (86.1)	[118]
67	3-OH	30.1 (-5.3)	[118]
68	3-CO_2CH_3, 6-F	40.4 (49.2); K_i=7.4 μM	[118]
69	3-CO_2CH_3, 6-Br	-4.9 (13.4)	[118]
70	3-CO_2H	K_i=40.6 μM	[118]
71	3-CO_2CH_3	K_i=259 μM	[118]
72	3-$CO_2CH_2CH_2CH_3$	K_i=98.0 μM	[118]
73	3-$CO_2C(CH_3)_3$	K_i=89.7 μM	[118]
74	3-$CH_2CH_2CH_2CH_3$	K_i=3.3 μM	[118]
75	3-NO_2	K_i=37.5 μM	[118]
76	3-$CO_2CH_2CH_3$, 6-F	K_i=21.0 μM	[118]
77	3-CO_2CH_3, 6-N=C=S	K_i=8.5 μM	[118]
78	1-CH_3, 7-OCH_3	10[b]	[113]
79	1-CH_3, 2-O, 7-OCH_3	46[c]	[113]
80	1-CH_3, 7-OH	-11[b]	[113]
81	1-CH_3	-13[b]	[113]
82	1- CO_2CH_3, 7- OCH_3	25[b]	[113]
83	1-CH_3, 7-OCH_3, 3,4-dihydro	4[b]	[113]
84	1-CH_3, 7-OH, 3,4-dihydro	21[b]	[113]
85	1,2,3,4-tetrahydro	0[c]	[113]
86	1-OH, 7-OCH_3, 3,4-dihydro	-13[c]	[113]

[a] Unless otherwise stated, inhibition data is reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Percent in parenthesis lists inhibition data with 2 h pre-incubation of inhibitor with IDO.

[b] 2 mM inhibitor concentration used in inhibition assay with rabbit intestine IDO.

[c] 1 mM inhibitor concentration used in inhibition assay with rabbit intestine IDO.

Table 5: β-Carboline Ring Substitution Compounds

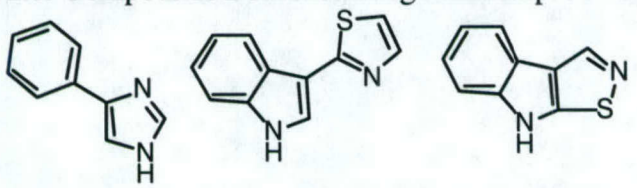
2.1.7 Miscellaneous Structures

A short group of other compounds have been discovered to be noncompetitive inhibitors. Although limited in number, these structures provide some unique and potent structural leads. 4-Phenylimidazole (**87**) is believed to bind to the heme iron similar to β-carboline (**59**)[25]. It seems possible that brassilexin (**89**) may also bind to the heme iron through the sulfur of the isothiazole ring.

Compound	Structure	Inhibition Data (%)[a]	Reference
87	4-phenylimidazole	K_i=4.4 μM	[117]
88	camalexin	21.3	[118]
89	brassilexin	K_i=5.4 μM	[118]

[a] Unless otherwise stated, inhibition data is reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor.

Table 6: Other Compounds Demonstrating Noncompetitive Inhibition



4-phenylimidazole camalexin brassilexin

2.1.8 Summation

Although a range of compounds have been investigated for IDO inhibition, submicromolar inhibition has not yet been achieved. A few unique structures have been discovered to have IDO inhibitory activity, nonetheless the majority of the most active structures contain the indole system or resemble L-Trp. Clearly, one important goal in the development of IDO inhibition as a cancer therapy will be to discover more potent inhibitors, and it seems that a diversification of IDO inhibitor structures may be necessary to achieve this goal.

2.2 IDO Inhibition *In vivo*

2.2.1 Pharmacology of IDO inhibitors. Until recently, 1MT has been the only compound evaluated as an IDO inhibitor *in vivo*. The means of delivery used in the majority of studies has been the same as that used in the original allogeneic conceptus rejection study [52]. This involves encapsulating the 1MT compound in a polymer matrix. The pellets (as prepared by Innovative Research, Inc.) are claimed to provide a steady state release rate of 10 mg/day, although no data directly demonstrating this has been provided. An alternative oral bolus dosing procedure for effectively delivering 1MT in the drinking water has been reported in one study [76]. However, 1MT is not, as claimed, soluble under the conditions described in the Methods.

During the course of tumor treatment studies, we have evaluated the serum levels of 1MT achieved with subcutaneous pellet implants. Two 140 mg pellets per mouse resulted in a steady state level of 1MT in the serum of 100 μM within 24 hours after implantation. At the published compound release rate these pellets were expected to maintain a constant level of serum 1MT for a period of 2 weeks. We found, however, that this steady state level of 1MT was maintained for only 5-7 days post-implantation after which time serum 1MT rapidly dropped to negligible levels.

1MT has been successfully prepared for bolus administration by oral gavage as a bead-milled suspension in methocel/tween (0.5% methylcellulose/1% tween 80). Pharmacokinetic (PK) analysis has revealed that 1MT has reasonable oral bioavailability and a relatively slow clearance rate [95], allowing the exploration of oral dosing regimens that maximize the anti-tumor efficacy of 1MT+taxol combination treatment. The PK profile for 1MT following oral delivery of

100mg/kg compared favorably to intravenous delivery of a bolus injection of 100mg/kg in solution (50% Cremaphor EL/50% ethanol with 3 μl/ml lactic acid and diluted 1:5 in sterile saline) [95] indicating that 1MT is orally bioavailable by this method of administration. The observed Cmax was 41μM at 1hr following oral administration of 100 mg/kg 1MT and the serum level declined by only ~3.3-fold by 8hr to 12.5 μM. By comparison, s.c. time-release pellets delivered 1MT at a steady-state dose of ~100 μM (data not shown). Titration of 1MT in the MMTV-*Neu* autochthonous breast cancer treatment assay indicated that reducing the dose of 1MT by half had no apparent effect on responses but a one quarter dose resulted in diminished efficacy (AJM, J DuHadaway, E Sutanto-Ward and GCP, unpublished data). These studies delineate an approximate minimal 1MT dose required to achieve regression of MMTV-*Neu* tumors in the two week assay. Oral dosing of 1MT at 400 mg/kg achieves a Cmax of ~225 μM (AJM, J DuHadaway, and GCP, unpublished data). Assuming a similar rate of clearance at this higher dose, the data are consistent with the expectation that a single oral dose can, for ~8 hr deliver, a level of compound that will produce efficacy (for 400mg/kg delivered p.o. the serum level at the 8hr timepoint is predicted to be ~68μM, which is higher than the estimated efficacious level of 50μM). Oral dosing on a bid schedule should therefore be sufficient to maintain an efficacious serum level in the mouse through trough. We have tested this prediction in the MMTV-*Neu* tumor treatment assay and shown that bid dosing of 1MT at 400 mg/kg for 5 days is at least as effective as the implanted pellets. This analysis argued that intermittent dosing can also effectively break tolerance when combined with chemotherapy. Reducing the dosing schedule to 4, 3, and 2 days resulted in a progressive loss of efficacy with decreased dosing (AJM, J DuHadaway, E Sutanto-Ward and GCP, unpublished data). The ability to effectively deliver 1MT in a controlled manner through oral administration will greatly facilitate further

acquisition of preclinical data regarding dosing and scheduling parameters for the development of an optimized combinatorial treatment protocol that will provide a necessary contextual framework within which to evaluate new more effective IDO inhibitors as are identified.

As noted in Secton 2.1.2, the thiohydantoin side chain modification of tryptophan produced a compound (MTH-trp) with 3-fold higher potency against purified human IDO enzyme than 1MT. The differential was even more pronounced in a cell-based assay, in which MTH-trp was demonstrated to be ~20x more potent than 1MT (EC50 = 12.5 μM for MTH-trp vs. >200 μM for 1MT; [95]). MTH-trp delivered in time release pellet format in combination with paclitaxel was at least as if not more effective than 1MT at producing regression of tumors in the MMTV-Neu model[95]. This demonstration of that structurally distinct IDO inhibitor exhibits a biological effect comparable to that of 1MT bolsters the argument that the proposed mechanism of action is correct. MTH-trp has not been evaluated for efficacy following oral gavage delivery because pharmacokinetic analysis has revealed that MTH-trp, unlike 1MT, is rapidly cleared from the bloodstream [95]. Thus, it appears unlikely that the efficacy achieved through oral gavage bolus dosing of 1MT in combination with paclitaxel can be replicated with MTH-trp.

2.2.2 Serum kynurenine as a biomarker for IDO activity.

Studies have reported the functional consequences of IDO inhibitor treatment without assessing IDO inhibition *in vivo* or correlating IDO inhibition with the observed biological consequences. This raises the legitimate concern of 'off target' effects (e.g. perhaps due to binding to other enzymes that bind tryptophan). The degradation of tryptophan by IDO produces N-formylkynurenine. This in turn is rapidly converted to kynurenine due to high levels of

kynurenine formamidase activity, the specific activity of which far exceeds that of IDO. However, tissues other than liver and kidney showed no significant activity associated with other key enzymes on the kynurenine pathway, and in mice the induction of IDO by systemic exposure to LPS coincided in large part with the change in plasma kynurenine level [41]. Levels of kynurenine as well as tryptophan in the serum can be simultaneously measured by HPLC-based analysis [123-125]. We are developing this procedure for use as a pharmacodynamic assay to evaluate inhibition of IDO *in vivo* by compounds administered to mice. Intraperitoneal administration of bacterial lipopolysaccharide (LPS) induces IDO activity in a variety of tissues resulting in the production of kynurenine and its release into the bloodstream. Peak kynurenine levels are reached one day after LPS administration[41,43]. The serum kynurenine pool is rapidly turned over with a half-life of <10 min [41,126], so pre-existing kynurenine should not mask the impact of IDO inhibitor treatment on kynurenine production. The serum level of compound being tested for IDO inhibitory activity can also be determined by HPLC analysis of the same sample, permitting concurrent collection of pharmacokinetic data from a single experiment.

In patients, determination of the kynurenine to tryptophan ratio has frequently been used to provide an estimate of IDO activity that is irrespective of the tryptophan baseline levels. which can be influenced to a minor extent by dietary uptake [127,128]. Measuring tryptophan and kynurenine levels in plasma or serum is much less invasive than enzyme activity tests performed on tissue samples. Thus, the serum kynurenine to tryptophan ratio can serve as a useful *in vivo* biomarker for evaluating IDO inhibitors in the clinic. The serum kynurenine to tryptophan ratio also correlates closely with neopterin concentrations for a variety of diseases [129]. IFNγ stimulates guanosine triphosphate (GTP) cyclohydrolase I the key enzyme for pteridine

production, and human monocytes/macrophages release large amounts of neopterin in response to IFNγ stimulation [130]. Therefore, although it is not directly linked to IDO activity, neopterin can serve as another sensitive biomarker for activation of cellular immunity.

CONCLUSION

From the standpoint of therapeutic development, IDO as a drug target offers a number of appealing features. First, as a single-chain catalytic enzyme with a well-defined biochemistry, IDO is highly tractable for inhibitor development compared to most other therapeutic targets in cancer. Second, the only other enzyme known to catalyze the same reaction, TDO2, has a much more restricted substrate specificity simplifying the problem of possible 'off target' effects. Third, the medicinal chemistry of indoleamines and indoleamine mimetics is well-developed. Fourth, lead inhibitors exist in 1MT and MTH-trp, both of which are bioactive and orally bioavailable. These inhibitors may offer tools for clinical validation of the novel combination principle reviewed here. Fifth, an *Indo* gene 'knockout' mouse has been reported to be viable and healthy [64], indicating that inhibitors will be unlikely to produce unmanageable mechanism-based toxicities, although promotion of inflammatory conditions remains a valid concern. Sixth, the combination of tryptophan and kynurenine, (the major substrate and downstream product of the IDO reaction, respectively) provides a useful biomarker for the pharmacodynamic evaluation IDO inhibitors. This analysis can be performed on blood samples, which eliminates the need to obtain and analyze tumor biopsy specimens that can be difficult, expensive, technically challenging, and troublesome to obtain from patients on trial. Lastly, small molecule immunomodulatory agents are likely to offer substantial logistical and cost advantages relative to both biologics and cell-based immunotherapies. IDO has clearly become established as an attractive and tractable target for the development of better small molecule inhibitors for possible use as immunomodulatory adjuvants to enhance standard chemotherapy and with perhaps the potential for even broader applicability for use against diseases characterized by immune suppression.

EXPERT OPINION

The successful treatment of advanced, metastatic cancers remains an elusive goal. Anti-cancer drug development still focuses predominantly on producing compounds that elicit direct cytotoxicity against tumor cells. Cytotoxic chemotherapeutic agents are currently among the most effective agents in the clinic but these compounds do not have favorable safety profiles. Recently, attention has been shifting towards finding drugs that target specific signal transduction pathways, but, at least so far, the therapeutic impact of such agents has been limited. A popular truism in the cancer field today is that specific combinations of targeted agents will have to be tailored to individual tumors based on their genetic makeup. Such balkanization of cancer therapy clearly runs counter to the contemporary managed care culture. This reality, in our opinion, means that the partitioning of cancer patients among a plethora of small niche markets is likely to be a prohibitively expensive proposition.

Strategies aimed at activating anti-tumor immunity have the potential to be useful against a wide variety of tumors and might be a particularly effective for treating disseminated metastases – the overarching problem facing cancer patients. On this basis, many novel anti-cancer therapies are currently being developed, with the purpose of stimulating immune responses through the use of cytokines, recombinant antibodies directed at tumor cells, anti-tumor vaccines, or cell-based, tumor-targeted immune therapies[131]. All these therapies rely on large, complex biologically-based agents or whole cells. Small molecules have clear advantages over biologics in terms of production, delivery, and cost. However, few small molecule agents for stimulating anti-tumor immunity have been described. In this regard, IDO represents a particularly appealing target. As a classical biochemical enzyme (a rarity amongst cancer targets), IDO is particularly well suited for pharmacological intervention, there being a wealth of medicinal chemistry knowledge and

experience in the successful development of such drugs. A further advantage stems from the determination that IDO inhibitors appear to work most effectively as immunomodulatory adjuvants for enhancing chemotherapy, as this suggests that use of IDO inhibitors might be successfully added to standard treatment protocols, which should ease their adoption into the clinic.

Like IDO, a number of other factors, upregulated at tumor sites, have been implicated in the establishment of tumoral immune escape including; transforming growth factor B, interleukin 10, prostaglandin E_2, Fas, tumor necrosis factor related apoptosis inducing ligand (TRAIL), and RACS1 to name a few. Why then focus on IDO? In large measure, it has been a case of the biology leading the way. Many of the same mechanisms implicated in tumoral immune escape are also posited to protect the developing fetus. However, the 1MT experiment [52] (which we have reproduced [95]) is so dramatic, that it strongly implicates IDO as being a particularly powerful, and in this case essential, mechanism for immune protection. Studying the mechanistic basis of how Bin1 loss contributes to tumorigenicity has further pointed towards the importance of IDO-mediated immune tolerization [95]. Reestablishing control over IDO that is lost in Bin1 null cells might even be a therapeutic alternative to direct inhibition of IDO activity, though how this might actually be achieved is as yet undetermined. Understanding how Bin1 exerts control over IDO is currently an area of active research.

Autochthonous MMTV-Neu tumors were found not to be as dependent on IDO activity for survival as the developing fetus. This was not altogether surprising given the weaker nature of the tumor antigens involved and the greater plasticity of cancer cells in responding to immunological pressure. The MMTV-Neu mouse mammary gland tumor model was specifically chosen for study because it is not artificially dependent on IDO for its outgrowth, but rather

mimics as closely as possible human disease. Conceptually it makes sense that the problem of immune escape might have to be attacked on multiple fronts to generate an effective anti-tumor response. What was perhaps counterintuitive, however, was the finding that standard cytotoxic chemotherapeutic agents show striking cooperativity. Therapies that combine immunotherapy and cytotoxic chemotherapy have not been widely explored, even in preclinical settings, perhaps because of the assumption that such combinations will work at cross purposes (since cytotoxic chemotherapy kills immune cells that immunotherapy targets for stimulation). Our findings argue that, on the contrary, immunotherapy can strongly promote the anti-tumor efficacy of chemotherapy. IDO inhibition was found to potentiate the anti-tumor efficacy of several DNA damaging agents as well as paclitaxel without elevating side-effects of these agents. While the mechanism underlying the observed pattern of cooperation remains to be defined, it is notable that IDO inhibition cooperated with all of the DNA damaging agents but none of the anti-metabolic agents tested. One interpretation of this pattern of cooperation is that IDO may facilitate tumor survival in response to certain kinds of genotoxic stress, perhaps by attenuating the immune response elicited by cells that display certain types of DNA damage, sustain certain checkpoint responses, or undergo certain kinds of cell death (e.g. apoptosis versus non-apoptotic cell death). A second interpretation is that the chemotherapies that cooperate with IDO inhibition are those that stimulate anti-tumor immunity in a complementary manner (i.e. combination therapy is composed of two immunotherapies). While there is extensive evidence that the efficacy of cytotoxic drugs is based in their ability to directly kill cancer cells, there is also evidence that many of these drugs can also stimulate anti-tumor immunity [97,98,101]. One appealing aspect of this idea is that it addresses what some might view as a paradox of our findings, namely, how a cytotoxic agent that kills immune cells might possibly cooperate with an

immune stimulatory agent. In future work, it will be important to rigorously test these two alternatives, for example, by determining whether combinatorial efficacy is retained against tumor cells that are resistant to chemotherapy-induced cell death, and whether the cytotoxic components of different effective combinations have mechanistically similar effects on the how the immune system responds to tumors despite mechanistic diversity in cytotoxic mechanisms.

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